REGULATION AND FUNCTION OF THE RHO GTPASE MEDIATED SIGNALING PATHWAYS IN METASTASIS AND LENTICULAR DIFFERENTIATION

A Dissertation

by

DIANNE COURTENAY MITCHELL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2006

Major Subject: Genetics

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Approved by:

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ABSTRACT

Regulation and Function of the Rho GTPase Mediated Signaling Pathways in Metastasis and Lenticular Differentiation. (May 2006)

Dianne Courtenay Mitchell, B.S., Oklahoma State University

Chair of Advisory Committee: Dr. Mingyao Liu

Modulation of the actin-based cytoskeleton and transcription factor regulation are merely two essential functions in a wide array of cellular activities that the Rho family of small GTPases is responsible for mediating. Aberrations in, or loss of, Rho GTPase signaling has been found to lead to multiple pathologies, including both metastatic progression and lenticular differentiation leading to cataractogenesis. This study has examined the transcriptional regulation of the metastasis suppressor, KiSS-1. Although the mechanism by which KiSS-1 modulates an anti-metastatic effect is not entirely known, it is known that KiSS-1 mediates stress fiber formation, increased adhesion and reduced migratory and invasive properties through modulation of the Rho family of small GTPases. The loss of KiSS-1 that commonly occurs during metastatic progression, leads to a loss of proper Rho GTPase regulation. This study has examined how KiSS-1 is regulated in two tissue types, breast and skin, and how the loss of AP-2α and DRIP-130, respectively, leads to the progression of breast cancer and melanoma. In addition, this study has also looked at the importance of Rac1 expression and function in the lens epithelium. Activation of Rac1 and its downstream effector, SRF, have been shown to be key regulators in lens cell differentiation, possibly leading to lens opacification via its transcriptional control of the

structural crystallins within the lens. The results of this dissertation research have made significant strides in understanding the nature of the anti-metastatic effects registered by the novel KiSS-1 peptide and its cognate GPCR. Additionally, it has shed light on the Rho family regulation of lens epithelial cell differentiation, indicating the elaborate involvement of Rac1 in mediating lens fiber development. In all, this research has determined previously unknown roles of small molecule GTPases in both the progression of metastasis, as well as in normal and abnormal lens cell differentiation.

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CHAPTER I

INTRODUCTION TO METASTASIS SUPPRESSOR KISS-1

Maintaining tight regulation of cellular activities including differentiation, proliferation and migration is essential in preserving a normal cell phenotype. This control is mediated through the association of extracellular molecules, including various growth factors, with their receptors in order to modify the intracellular signal transduction pathways that affect cellular changes. Normal cells exhibit a highly dynamic nature; regulation is maintained by switching from one pathway to the next as exposure to different environmental stimuli varies. Mutations of genes encoding cell cycle regulators or proto-oncogene such as ras, Src, and epidermal growth factor receptor or to tumor suppressor genes, tip the balance and induce transformation to an immortalized, unregulated cancer cell phenotype. Cancers most commonly result from the compounding of multiple genetic mutations that lead to aberrations in essential cell function (Bieche and Lidereau, 1995).

The vast majority of breast cancer deaths occurring each year result from complications caused by tumor cell metastasis, rather than as a consequence of the original tumor. Once tumorigenic cells enter into the vascular and lymphatic systems, they travel

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This dissertation follows the style of *Cell*.

peripheral regions where they invade tissues and form neoplasms. Metastasis is a process requiring detachment of cancer cells from the primary site, survival of sheer forces encountered in the circulation, migration to other organs, attachment to and invasion of tissues, proliferation of these cells at the secondary site, and finally the capacity to enlist neighboring capillaries to supply the tumor with nutrients as it develops. Thus, migration, invasion and angiogenesis are as crucial to tumor formation and metastasis as they are to normal cell growth and proliferation. Interference at any one of these steps can block this metastatic cascade thereby preventing the formation of metastatic growths. Consequently, there is an increasing interest in studying the metastatic process in order to identify possible ways to inhibit its progression. Our laboratory, and other groups have determined that the KiSS-1 peptide inhibits cell migration and invasion, thus interfering with the metastatic cascade and preventing tumor metastasis.

Research has also shown that the expression of the KiSS-1 peptide, like many other tumor suppressor genes, is reduced or absent in breast cancer metastases. Unlike tumor suppressor genes, metastasis suppressors like KiSS-1 function by inhibiting metastasis of cancer cells to secondary sites, therefore abrogating the spread of cancer. Most metastasis inhibitors have only been identified over the past 5-10 years, and the mechanisms through which they modulate their anti-metastatic effects have not yet been determined. It is known, however, that the effects of KiSS-1 are mediated through its cell surface receptor protein, the KiSS-1 receptor which transduces the presence of the peptide into downstream signaling events. Experiments have shown that overexpression and activation of the KiSS-1 receptor by its ligand in highly metastatic breast cancer cells permits cells to recover

their metastatic inhibition. Similar to that of other metastasis suppressors, the inverse correlation of KiSS-1 receptor activation with a higher incidence of breast cancer metastasis is clear, although the mechanism of this association remains obscure (Shevde and Welch, 2003; Steeg et al., 2003). Our laboratory has begun to characterize the mechanism by which the KiSS-1 receptor elicits its anti-metastatic effect, principally through close examination of its downstream signaling pathways and through understanding of the mechanisms controlling its transcription. This research will help to determine the overall effects modulated by the activated KiSS-1 receptor and to identify which signaling pathways are important for conveying the anti-metastatic effect of activated KiSS-1 receptor in breast cancer cells. By gaining a better understanding of the metastatic cascade and the role activated KiSS-1 receptor plays in preventing breast cancer metastases, this research may lead to new methods of breast cancer intervention and provide new means of breast cancer prevention.

G-protein coupled receptors (GPCRs) are integral membrane proteins that bind specific external ligands. Upon ligand binding, GPCRs transduce signals across the membrane via activation of heterotrimeric GTP-binding proteins, which initiate an intracellular cascade of signaling events. GPCRs are composed of seven transmembrane regions, and upon activation, either the intracellular loops or carboxy-tail mediate G-protein interaction. The heterotrimeric G-protein complex consists of α, β and γ subunits which modulate distinct internal signaling pathways (Gudermann et al., 1997). G-proteins are intrinsic to JNK, MAPK and PLC-β signaling pathways, indicating they play an important role in cytoskeletal reorganization, migration, and invasion (Gutkind, 1998).

Subsequently, overexpression of some GPCRs has been directly linked to the onset of cancers (Dhanasekaran et al., 1995; Zohn et al., 2000).

Like other GPCRs, the KiSS-1 receptor transduces the presence of an external stimulant to the interior of the cell, thus initiating a series of internal signaling cascades that mediate the effects of that stimulant. In the case of the KiSS-1 receptor, the stimulant is the KiSS-1 peptide, and the signaling events induced by of this peptide lead to the suppression of breast cancer metastasis. The KiSS-1 peptide has been recognized as a potent metastasis suppressor in previous studies (Lee et al., 1996; Lee and Welch, 1997a). Its anti-metastatic properties were identified through subtractive hybridization analysis during which microcell-mediated replacement of KiSS-1 expression in C8161 melanoma cells suppressed melanoma metastasis by 95% as compared to untreated melanoma (Lee et al., 1996).

Breast cancer cells commonly show a loss of various chromosomal regions resulting in loss of heterozygosity. The region on which the KiSS-1 peptide resides (chromosome 1q) is one of these chromosomal regions that are frequently lost during breast cancer development. Reintroduction of KiSS-1 expression in human breast carcinoma cell lines (MDA-MB-435) results in the dramatic suppression of metastasis (Lee and Welch, 1997a). Similar *in vivo* studies showed that injection of metastatic cells over-expressing KiSS-1 peptide into the mammary fat pads of athymic nude mice resulted in decreased metastasis of the breast cancer to the lung or lymph nodes (Lee and Welch, 1997b).

It is the activation of the KiSS-1 receptor that conveys the presence of the peptide and initiates those signaling events that eventuate metastatic inhibition of breast cancer. Upon activation of the receptor by its ligand, the KiSS-1 receptor increases internal calcium levels in Chinese hamster ovary cells, suggesting a strong signaling response within the cell (Ohtaki et al., 2001). Likewise, activated KiSS-1 receptor mediates the excessive formation of focal adhesions and stress fibers, indicating increased adhesion and decreased migratory and invasiveness of these cells (Ohtaki et al., 2001). This data suggests that activation of KiSS-1 receptor may induce an excessively adhesive phenotype which results in a loss of cell motility.

Although the correlation between increased activation of KiSS-1 receptor and increased metastatic inhibition in cancer cells is clear, the mechanism through which activated KiSS-1 receptor prohibits breast cancer metastasis remains unknown. Our laboratory has recently cloned the human and mouse isoforms of KiSS-1 receptor, as well as its ligand (Stafford et al., 2002). The mouse forms of KiSS-1 receptor and peptide show a high degree of homology, indicating that they have been well conserved over the course of evolution in order to serve a necessary molecular function. In our preliminary experiments, we have shown that activation of KiSS-1 receptor by its ligand can inhibit cell proliferation, migration, and invasion in NIH3T3 cells (Stafford et al., 2002).

Because the metastatic cascade is composed of many separate and distinct processes (migration, adhesion, invasion, etc.), many signaling pathways are involved in its overall regulation. GTPases, proteins that regulate G-protein coupling and membrane receptor activity, are commonly involved in tumorigenesis and have been shown to elicit a

substantial effect on cancer metastasis (Figure 1). Suppression of either Cdc42 or Rac leads to decreased cell spreading and decreased tumor metastasis. Migration, invasion, and vascularization require clustering of basement membrane-associated integrins and the recruitment of cytoskeletal adaptor proteins such as focal adhesion kinase (FAK). Integrins are transmembrane glycoproteins that interact with extracellular matrix proteins to induce mitogenic, chemotaxic and adhesive functions within the cell (Rolli et al., 2003). FAK influences migration, proliferation and cell survival changes and activates Rac and JNK (Hsia et al., 2003) promoting tumor cell metastasis. Vascular endothelial growth factor (VEGF) induces the proto-oncogene Src to associate with FAK to promote cell invasion, angiogenesis and migration (Hsia et al., 2003). Our laboratory has recently shown that active FAK is downregulated upon overexpression of activated KiSS-1 receptor, suggesting a possible mechanism by which KiSS-1 receptor modulates its anti-metastatic properties (Stafford et al., unpublished data).

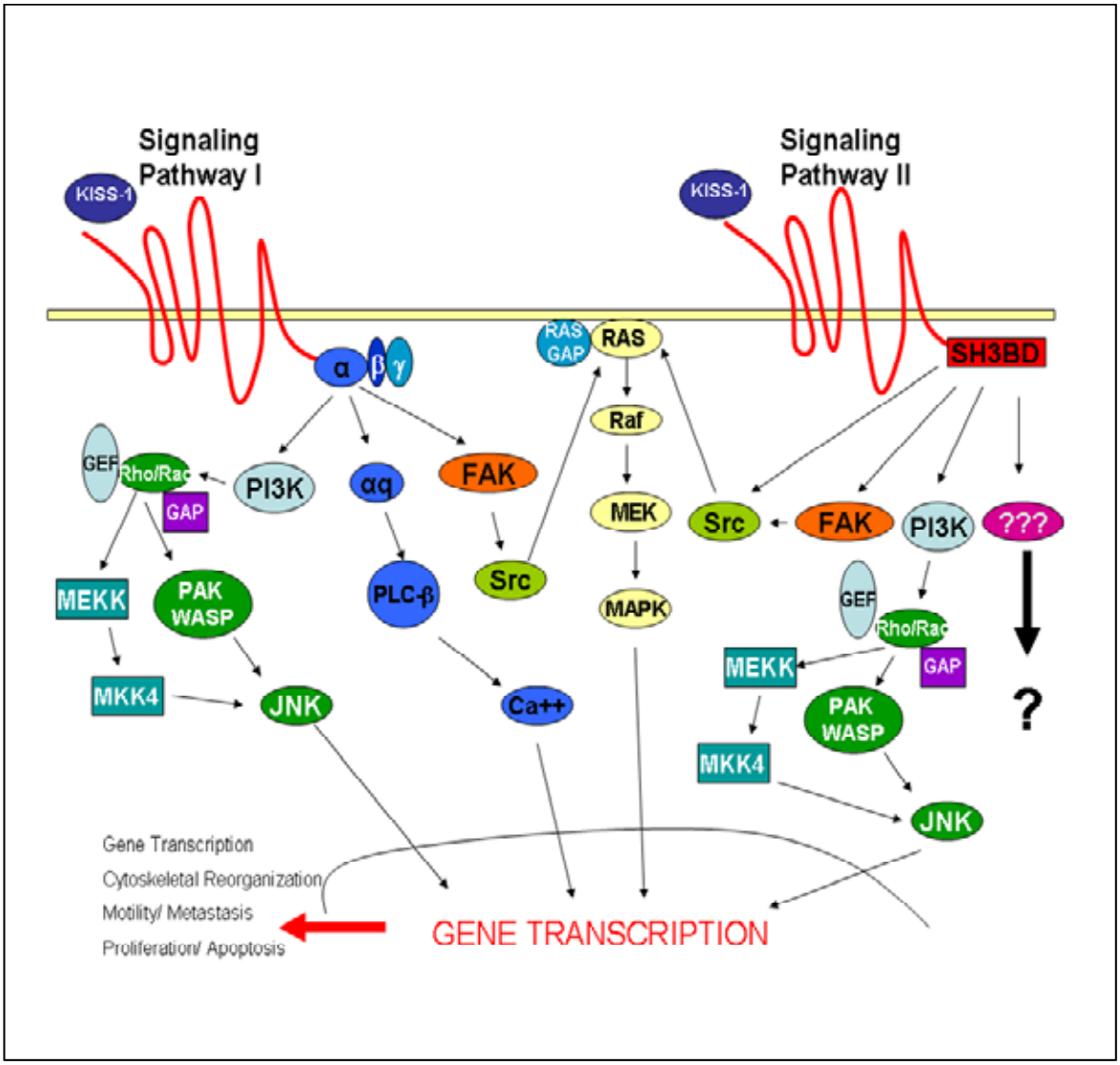

Figure 1: Possible KiSS-1 Mediated Signaling Pathways

CHAPTER II

MATERIALS AND METHODS

Cell Culture and Cell Transfection

MCF-7, MDA-231, MDA-435, and T47D cells were obtained from the American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium and RPMI 1640 with phenol red, 100X antibiotics, and fetal bovine serum were purchased from HyClone (Logan, UT). Media for melanoma lines (A375SM, MeWo, SB2, and WM2664) was supplemented with Hepes Buffer (Hyclone; Logan, UT) and 100X MEM Non-Essential Amino Acids (Gibco; Grand Island, NY). Transfection of both breast and melanoma cell lines was carried out according to the manufacturer's protocol (Invitrogen). In brief, DNA was added using a Lipofectamine to DNA ratio of 2:1 to each well of a 6- or 24-well plate or 100-mm dish for a period of 6 hours. Empty vector was used to offset the difference in DNA concentrations in reactions in which fewer test plasmids were transfected. Transfection reagent was then removed from each well, and cells were incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for 24 hours before assaying.

Chemicals, Constructs, and Oligonucleotides

[γ-32P]ATP (300 Ci/mmol) was obtained from PerkinElmer Life Sciences (Wellesley, MA). Poly(dI-dC) and T4 polynucleotide kinase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Antibodies for Sp1, AP-2α, IgG, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Luciferase reagent and

lysis buffer were obtained from Promega Corp. (Madison, WI). The 1.2-kb KiSS-1 promoter was cloned from BAC clone RP11–203F10 (accession AL592114) using primers consisting of XhoI and KpnI sites for ligation into the pGL3-basic vector from Promega Corp. (Madison, WI); sense, 5'-GGGGTACCAGACTGCCGGCATGCTT-3' and antisense, 5'-CCGCTCGAGTT-CTCCCCAGCTCCCTGATCACATCC-3'. All other KiSS-1 promoter mutants and truncated fragments were likewise cloned into XhoI and KpnI sites in the pGL3-basic vector. Expression vectors for AP-2α, AP-2B, and Sp1 were cloned as previously described (Tellez and Bar-Eli, 2003). The Sp1-ΔDBDconstruct was cloned into pCDNA3.1 (Invitrogen) and was generated from the full-length Sp1 construct with PCR primers, which excluded the DNA-binding region; sense, 5'-CGGAATTCATGAGCG-ACCAA-GATCACTCCATGGATC-3' and antisense, 5'-CCGCTCGAGGAAGCCA-TTGC-CACTGATATTAAT-3'. Additionally, antibodies for DRIP-130 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The DRIP-130 construct was provided by Dr. Jun Qin (Baylor, Houston, TX).

Luciferase Assay

Cells were cultured in 24-well plates in Dulbecco's modified Eagle's medium or RPMI 1640 supplemented with 10% fetal bovine serum. After 18–20 h when cells were ~60% confluent, reporter gene constructs were transfected using Lipofectamine reagent according to the manufacturer's protocol (Invitrogen). In brief, 1 μg of total DNA was transfected into each well of a 24-well plate using a Lipofectamine to DNA ratio of 2:1 for a period of 6 hours. Empty vector was used to offset the difference in DNA concentrations in reactions in which fewer test plasmids were transfected. Transfection reagent was then

removed from each well, and cells were incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were harvested after 48 hours, and luciferase activity of protein lysates was measured following the manufacturer's protocol (Luciferase Assay System, Promega). To normalize for differences in cell line transfection efficiencies, all cells were transfected with pRSV-β-gal control vector (Promega). β-Galactosidase levels were then measured following the manufacturer's protocol (Galacto-Light Plus, Bedford, MA).

Western Immunoblot Analysis

Breast and melanoma cell lines (2.0×10^7) were seeded in 100-mm Petri dishes with 10 ml of complete medium and incubated overnight. The cells were then scraped off and washed in cold phosphate-buffered saline. The cell pellet was then lysed in 0.5 ml of RIPA buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, 100 units/ml aprotinin). Soluble proteins were then separated by centrifugation at 15,000 μ*g* for 5 minutes at 4 °C. Protein concentration was determined. Samples were then diluted into loading buffer at 1 mg/ml. Following heat denaturation, samples containing 10 μg of protein were loaded onto and separated on 10% or 15% SDS-PAGE gels as needed. Proteins were then transferred electrophoretically to 0.45-μm nitrocellulose membrane (Pall Corp., Pensacola, FL). After incubating the membranes in blocking solution, primary antibody was added at 1:1,000 dilution, followed by secondary antibody incubation at 1:10,000. Proteins were detected using the SuperSignal West Pico Chemiluminescent substrate (Pierce) according to the manufacturer's instructions.

To extract total proteins from the cornea, retina, or the whole lens, different part of the mouse eye tissues were dissected out and grinded under liquid nitrogen to make a fine powder and suspended in cell lysis buffer (Li et al., 2001). The insoluble material was subsequently removed by centrifugation. For each sample, the protein concentration was determined as previously described (Li et al., 2001). Western blot analysis of total proteins was conducted as described before (Li et al., 2003). Briefly, 100 ng of total proteins in each sample were resolved by 15% SDS-polyacrylamide gel. The protein blots were blocked with 5% milk in TBS (10 mM Tris·HCl, pH8.0/ 150 mM NaCl) overnight at 4^{o}C, and incubated with primary antibodies as described above. Following incubation with the primary antibody, the membrane was exposed to a horseradish peroxidase conjugated secondary antibody, subjected to SuperSignal West Pico Chemiluminescent reagent (Pierce Biotechnology, Inc.), and exposed to film. A single protected band at approximately 21 kDa was observed for each GTPase-specific antibody used.

Electrophoretic Mobility Shift Assay (EMSA)

For electrophoretic mobility shift assay (EMSA), nuclear extracts from MCF-7, A375SM and WM2664 cells were harvested as described previously (Abdelrahim et al., 2004). Protein concentrations of nuclear extracts were determined using BCA assay (Pierce). Aliquots of nuclear protein were frozen and stored at -80 °C until used. *KiSS-1* promoter-derived oligonucleotides were synthesized and annealed, and 5 pmol was 5'-endlabeled using T4 Kinase and [γ32-P]ATP. A 30-µl EMSA reaction containing ~100 mM potassium chloride, 3 µg of crude nuclear extract, 1µg of poly(dI-dC) with or without unlabeled competitor oligonucleotide, and 10 fmol of labeled probe was incubated on ice

for 20 minutes. A Sp1-specific antibody was then incubated in appropriate reactions for 20 min on ice. A separate AP-2 antibody was used for gel-shift analysis (Active Motif, Montreal, Canada). DNA-protein complexes were then resolved on 5% PAGE gel at ~120 V at room temperature for 2 hour. Antibody-protein complexes were observed as supershifted or immuno-depleted complexes.

Chromatin Immunoprecipitation (ChIP) Assay

Chromatin immunoprecipitation (ChIP) was performed following the protocol outlined by the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). Briefly, MCF-7 cells transfected with both AP-2α and Sp1 (2×10^7 cells) were fixed with 1% formaldehyde, scraped into conical tubes, pelleted, and lysed in SDS lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1μg/ml pepstatin A. DNA was sheared to fragments of 200–500-bp by eight 10 second sonications. The chromatin was precleared with salmon sperm DNA/protein A-agarose slurry (Upstate Biotechnology) for 1 h at 4 °C with gentle agitation. The agarose beads were pelleted, and the precleared supernatant was incubated with antibodies to IgG, AP-2α, and Sp1 overnight at 4 °C. The region between +288 and +188 of the KiSS-1 promoter was amplified from the immunoprecipitated chromatin using the following primers: sense, 5'-ATAGCCCATTTCCACGTTG-3' and antisense, 5'-GGCGGGACTTTCTCCTTC-3'. Following PCR, the 100-bp product was resolved on a 2.5% agarose gel and stained with ethidium bromide. Samples were visualized under UV light. Additionally, chromatin immunoprecipitation (ChIP) was performed using A375SM and WM2664 cells transfected with both DRIP-130 and Sp1 as previously described (Mitchell et al., 2006). The

precleared supernatant was incubated with antibodies to IgG, DRIP-130, and Sp1 overnight at 4 °C. The region between +1 and +156 of the KiSS-1 promoter was amplified from the immunoprecipitated chromatin using the following primers: sense, 5'-TTCTCCCCAGCTCCCTGATCACATCC-3' and antisense, 5'-CTGCCTCCAGT-CACAGAGC-3'. Following PCR, the ~150-bp product was resolved on a 2.5% agarose gel and stained with ethidium bromide. Samples were visualized under UV light.

Co-Immunoprecipitation Analysis

Interactions of AP-2α and Sp1 in transfected MCF-7 cells were examined by immunoprecipitation (IP) and by western blot analysis. Briefly, cells were lysed with RIPA buffer containing 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 10 mg/ml phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, and 1 mg/ml leupeptin, followed by sonication with a 550 Sonic dismembrator (Fisher Scientific) and immunoprecipitated with the indicated antibodies. A anti-AP-2α and anti-Sp1 immunocomplexes were recovered by using protein A beads (Sigma). All immunoprecipitates were washed with lysis buffer (4X) and were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore). After incubation in TBST buffer (20 mM Tris-HCl, pH 7.5/500 mM NaCl/0.02% Tween 20) containing 0.2% bovine serum albumin and 5% dry milk powder for 2 hours, the membranes were probed with the indicated antibodies and visualized with the SuperSignal West Pico detection system (Pierce).

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from breast cancer and melanoma cell lines with TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase and oligo(dT) (Promega) according to the manufacturer's protocol. Primer sequences used for detection of *KiSS-1* transcripts were 5'-GCCCACCATGAACTCACTG-3' and 5'-CTGC-CCCGCACCTGCG-3'. Amplified products were ~400 bases in length. Additionally, primers for β-actin were 5'-GGCTCCGGCATGTGCAAGGC-3' and 5'-AGATTTTCTCCATGTCGTCC-3', which resulted in PCR products of ~200 bases. Optimal PCR cycles required for linear amplification for each set was determined. β-Actin required 21–23 cycles per reaction, whereas KiSS-1 required 24–28 cycles. PCR products were separated using agarose gels of appropriate concentration, visualized by EtBr staining and quantitated using Alpha Imager software (Alpha Innotech, San Leandro, CA).

Invasion and Migration Assays

A375SM and WM2664 melanoma cell lines were transiently transfected with both DRIP-130 and Sp1 or with vector alone. Transfection efficiency was determined visually upon transfection of a GFP-tagged vector. As described earlier, cell migration assays were carried out in modified Boyden chambers (Banyard et al., 2000; Stafford et al., 2002). Briefly, the outside of the filters were coated with either 1 μg/ml collagen for 1 hour and then washed three times with PBS. Filters were then incubated with DMEM with BSA for 1 hour. Filters were then put into DMEM medium without FBS and with 0.5 ng of mouse bFGF. Both melanoma lines were seeded at approximately 20,000 cells/well on top of the

filter. Plates were incubated 18 hours. Excess cells that did not migrate through the filter were removed from the inside of the filter. Cells were fixed with 4% paraformaldehyde for 20 minutes, washed three times with PBS, and then stained with crystal violet. Stained cells were examined and counted under the microscope. Additionally, scratch assays were carried out on Sp1/DRIP-130 co-transfected A375SM and WM2664 cells. Cells were allowed to grow to confluency on plates coated with collagen and washed twice with PBS. Cells were then scratched with a pipette tip and washed five more times with PBS. Fresh DMEM was added with 0.5 ng of mouse bFGF. This was allowed to incubate for 24 hours and pictures were taken using a Nikon digital camera.

Animals

The C57BL/6 mice were used in all experiments. Animals were maintained in a 12 h light/dark cycles and fed normal diet and water. Mice used in this study were handled in compliance with the "Guide for the Care and Use of Laboratory Animals" (National Academy Press) and also with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Embryo Sections

Ocular tissues were fixed in Zinc-formalin fixative and processed by Excalibar Pathology (Moore, Oklahoma). The slides were deparaffinized with xylene, rehydrated, bathed in 3% H_2O_2 at 37 °C to quench the endogenous peroxidase, and protein antigens were unmasked by steaming slides in citrate solution for 45 minutes.

Immunohistochemistry

Immunohistochemistry (IHC) of paraffin embedded tissues was performed using the ABC-Staining System according to manufacturer's instructions (Santa Cruz Biotechnology). All sections were counterstained with Harris Modified Hematoxylin with acetic acid (Fisher Scientific). Polyclonal antibodies to RhoA, Rac1 and Cdc42 were obtained from Santa Cruz Biotechnology. Primary antibodies were applied at a 1:100 dilution for IHC. Control IHC experiments were performed using the rabbit pre-immune, and GTPase-specific antibodies blocked with saturating levels of corresponding RhoA, Rac1 or Cdc42 peptide antigen.

CHAPTER III

REGULATION OF KISS-1 METASTASIS SUPPRESSOR GENE EXPRESSION IN BREAST CANCER CELLS BY DIRECT INTERACTION OF TRANSCRIPTION FACTORS ACTIVATOR PROTEIN-2α AND SPECIFICITY PROTEIN-1*

Overview

KiSS-1 has been shown to function as a tumor metastasis suppressor gene and reduce the number of metastases in different cancers. The expression of KiSS-1 or KiSS1, like other tumor suppressor, is commonly reduced or completely ablated in a variety of cancers via an unknown mechanism. Here we show that the loss of KiSS-1 expression in highly metastatic breast cancer cell lines correlates directly with the expression levels of two transcription factors, activator protein-2α (AP-2α) and specificity protein 1 (Sp1), which synergistically activate the transcriptional regulation of KiSS-1 in breast cancer cells. Although the *KiSS-1* promoter contains multiple AP-2α binding elements, AP-2α-mediated regulation occurs indirectly through Sp1 sites, as determined by deletion and mutation analysis. Overexpression of AP-2α into highly metastatic breast cell lines did not alter *KiSS-1* promoter-driven luciferase gene activity. However, co-transfection of AP-2α wild-type or the dominant negative form of AP-2 lacking its C-terminal DNA-binding domain, AP-2B, together with Sp1, increased KiSS-1 promoter activity dramatically, suggesting that AP-2α regulation of *KiSS-1* transcription does not require direct binding to

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Sp1 to form transcription complexes at two tandem Sp1-binding sites of the promoter to activate *KiSS-1* transcription. Together, our results indicate that AP-2αand Sp1 are strong transcriptional regulators of KiSS-1 and that loss or decreased expression of AP-2α in breast cancer may account for the loss of tumor metastasis suppressor KiSS-1 expression and thus increased cancer metastasis.

Introduction

The vast majority of breast cancer deaths results from complications caused by tumor cell metastasis rather than as a consequence of the original tumor growth. Once tumorigenic cells enter into the vascular and lymphatic systems, they travel to peripheral regions where they invade tissues and form neoplasms. Metastasis is a process requiring detachment of cancer cells from the primary site, survival of sheer forces encountered in the circulation, migration to other organs, attachment to and invasion of tissues, proliferation of these cells at the secondary site, and finally the capacity to enlist neighboring capillaries to supply the tumor with nutrients as it develops (Pantel and Brakenhoff, 2004). Interference at any one of these steps can block this metastatic cascade thereby preventing the formation of metastatic tumor growths. Consequently, there is a growing interest in researching the metastatic process to identify possible ways to inhibit its progression.

Metastasis suppressor genes, which inhibit the spread of cancers to secondary sites, have become the target of mounting clinical and basic cancer research. One such gene, *KiSS-1* or *KiSS1*, was originally identified as a metastasis suppressor by microcell-mediated transfer in melanoma lines, by which it was found to reduce tumor cell invasive

and migratory properties without affecting their tumorigenicity (Lee and Welch, 1997a). Since then, KiSS-1 has been shown to act as a potent anti-metastatic agent either by treatment using synthesized KiSS-1 peptide or upon ectopic expression in highly metastatic cells (Lee and Welch, 1997a; Masui et al., 2004; Ohtaki et al., 2001; Lee and Welch, 1997b). Loss or reduced expression of KiSS-1 has been found in a variety of tumor metastasis, including breast cancer, bladder cancer, pancreatic cancer, and esophageal squamous cell carcinoma (Masui et al., 2004; Lee and Welch, 1997b; Ikeguchi et al., 2004; Sanchez-Carbayo et al., 2003). Together, these studies suggest that KiSS-1 is a human metastasis suppressor gene and that loss of KiSS-1 and its receptor may correlate with human tumor progression and metastasis.

The *KiSS-1* gene encodes a largely hydrophobic 145-amino acid protein highly expressed in the placenta (Ohtaki et al., 2001; Muir et al., 2001). The *KiSS-1* gene product consists of a protein kinase phosphorylation domain, a secretory signal, and polyproline-rich region, and a number of potentially important motifs for post-translational modifications (Muir et al., 2001; Lee et al., 1996). Independently, three groups discovered and isolated the 54-amino acid C-terminally amidated fragment of KiSS1 protein (amino acids 68–121), termed metastin and kisspeptin, respectively (Ohtaki et al., 2001; Kotani et al., 2001). The function of KiSS-1 peptide is mediated through interaction with the membrane-bound G-protein-coupled receptor, GPCR54, a close relative of the galanin receptor (Ohtaki et al., 2001; Masui et al., 2004; Kotani et al., 2001; Lee et al., 1999; Clements et al., 2001). C-terminal amidation of the KiSS-1 peptide leads to strong binding with GPCR54, initiating a series of cellular changes, including increased intracellular

[Ca2+] and inositol 1,4,5-trisphosphate release, as well as morphological changes, such as up-regulating focal adhesion and stress fiber formation (Ohtaki et al., 2001; Muir et al., 2001; Kotani et al., 2001; Stafford et al., 2002). Collectively, these signaling events inhibit chemotaxis and invasion, reducing the incidence of tumor metastasis (Ohtaki et al., 2001; Lee and Welch, 1997b; Stafford et al., 2002). However, while much has been learned about the possible physiological effects of KiSS-1 expression in cancer cells, the mechanism controlling KiSS-1 transcriptional regulation is still unknown as is the underlying reason for its loss during metastatic progression.

As breast cancer cells become increasingly metastatic, expression levels of different genes, contributing to cell cycle, tumor cell invasion, and migratory properties, are altered. Genes commonly found up-regulated in breast cancer, including cyclin D1, c-myc, and MMP-9, mediate behavioral and proliferative changes that stimulate oncogenesis, whereas the loss of c-Kit or p53 may also encourage an increased metastatic phenotype, because they are no longer present to block formation of neoplasms (Gillet et al., 1996; Yu et al., 2001; Wang et al., 2005; Stuelten et al., 2005; Smith et al., 2003; Huang et al., 1998). Transcription factors that modulate the expression of oncogenes play important roles during tumorigenesis and metastasis. AP-2α, is a 52-kDa transcription factor, regulates genes that are important during development and metastatic processes (Zhang et al., 1996; Bar-Eli, 1999). Genes under AP-2-modulated transcriptional regulation contain the consensus palindromic sequence 5'-GCCNNNGGC-3' and have a variety of cellular functions, such as human proenkephalin, plasminogen activator inhibitor type I, c-erbB-2, c-Kit, thrombin receptor, and vascular endothelial growth factor (Huang et al., 1998; Bauer

et al., 1994; Hyman et al., 1989; Descheemaeker et al., 1992; Bosher et al., 1995; Tellez et al., 2003). Located on the short arm of chromosome 6, AP-2 protein contains a DNA-binding domain, a protein kinase A phosphorylation site, and a transactivation domain (Gaynor et al., 1991; Williams and Tjian, 1991). A naturally occurring splice variant, AP-2B, lacks the DNA-binding domain, thus enabling it to function as a dominant negative form of the AP-2α protein (Gershenwald et al., 2001). AP-2 has been shown to regulate neoplasm development by directly and indirectly regulating gene expression. In addition to inducing or repressing activity of cancer-related genes at their promoter, AP-2α can physically interact with oncogenes, such as β-catenin, DEK, and Pax6, and is thereby directly involved in tumorigenesis and development (Turner et al., 1998; Zhang et al., 2003; Sivak et al., 2004; Li and Dashwood, 2004; Campillos et al., 2003; Nottoli et al., 1998). Loss of AP-2 is common in breast cancer and in many other cancers, resulting in the loss of regulation of multiple oncogenes and increased tumorigenesis. Sp1 has also been shown to regulate genes involved in tumorigenesis, including the up-regulation of both hepatocyte growth factor receptor and vascular endothelial growth factor (Liang et al., 2004; Finkenzeller et al., 2004; Abdelrahim et al., 2004; Yao et al., 2004). Additionally, Sp1 can directly interact with the c-Jun transcription factor to modulate the up-regulation of vimentin, a protein commonly found misregulated in metastatic tumors (Wu et al., 2003). Sp1 itself has been shown to be a useful molecular marker is gastric cancer and Sp1 expression has been shown to be closely associated with patient survival rate (Wang et al., 2005, Wang et al., 2003). Here, we report that the expression of KiSS-1 metastasis suppressor gene in breast cancer cells is directly correlated with the expression of

transcription factors AP-2α and Sp1, and that AP-2α and Sp1 synergistically activate the transcriptional regulation of KiSS-1 in breast cancer cells. Furthermore, we demonstrate that KiSS-1 expression is modulated by AP-2α through direct interaction with the transcription factor Sp1 at two tandem Sp1-binding sites rather than via interaction with the consensus AP-2-binding sites of *KiSS-1* promoter. These results offer a mechanism for the loss of *KiSS-1* gene expression commonly seen in metastatic breast cancers and provide another molecular mechanism by which AP-2α and Sp1 transcription complex modulates tumorigenesis and tumor progression.

Endogenous KiSS-1 Expression in Breast Cancer Cell Lines Correlates with AP-2α Expression Levels

We and other laboratories have reported that KiSS-1 expression is lost in highly metastatic breast cancer cells. To determine if loss of the transcription factor AP-2α and Sp1 was directly or inversely associated with loss of KiSS-1, the expression levels of AP-2α and Sp1 levels in both highly metastatic and non-metastatic breast cells were compared with the expression of KiSS-1 (Figure 2). Due to the lack of a specific and effective KiSS-1 antibody, KiSS-1 expression level in the breast cell lines was quantitated using RT-PCR and normalized to the β-actin level within each sample. The relatively non-metastatic breast cell line, MCF-7, showed dramatically higher -fold expression as compared with the more metastatic breast tumor cell lines (T47D, MDA-231, and MDA-435). Similarly, Western blot analysis demonstrated that AP-2α expression was lost or barely detectable in the metastatic cell lines, MDA-231 and MDA-435 (Figure 2). The expression levels of Sp1 were also examined in these cells. As shown in Figure 2 (*bottom*), Sp1 expression was

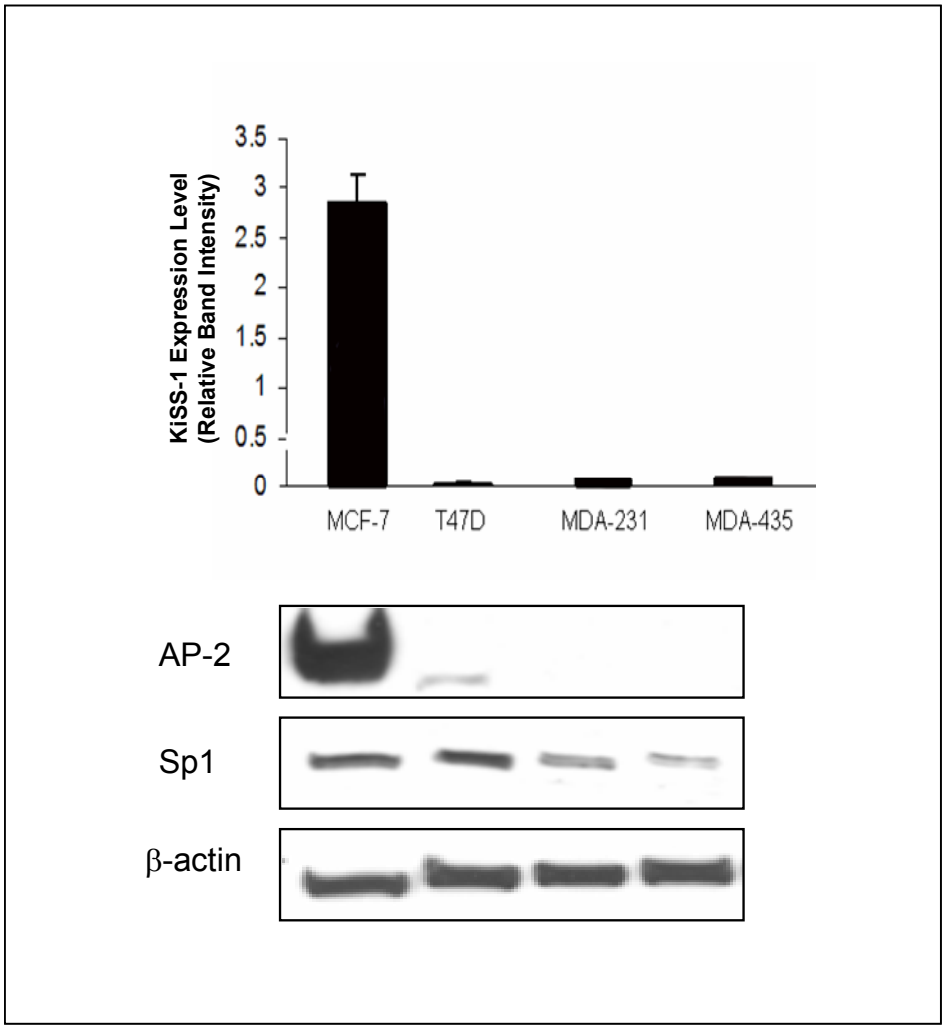

Figure 2: Western Blot and RT-PCR Analysis Correlating KiSS-1 Expression and AP-2α and Sp1 in Breast Cancer. RT-PCR and immunoblot analysis for the expression of KiSS-1, AP-2α and Sp1 in breast cancer cell lines. Highly metastatic breast cancer cell lines (T47D, MDA-231 and MDA-435) expressed low to undetectable levels of KiSS-1 and AP-2α, while the less metastatic MCF-7 cell expressed appreciably higher levels of both.

slightly decreased in the metastatic cancer cell lines (Figure 2). Together, these data suggest that the expression of KiSS-1 was correlated very well with the expression levels of the two transcription factors, especially AP-2α, in breast cancer cells. To further confirm that KiSS-1 expression is regulated by AP-2α and Sp1, we reintroduced both AP-2α and Sp1 into these cells (Figure 3). Activation of the *KiSS-1* promoter by overexpression of AP-2α and Sp1 was enhanced considerably from 4- to 27-fold depending on the cell line. Although it is not clear why transfection of AP-2α and Sp1 strongly activate *KiSS-1* promoter construct in MCF-7 cells where these factors are expressed at higher endogenous level, we speculate that overexpression of these two proteins have an additive effect on the activation of the KiSS-1 promoter construct with the endogenous factors. Therefore, we concluded that the loss of both AP-2α and Sp1 strongly and directly correlated with the loss of KiSS-1 expression in breast cancer cells.

Identification of the Regulatory Region of the KiSS-1 Gene by AP-2α and Sp1

Regions of sequence conservation, found between humans and mice or rats, are commonly used as an indicator of whether a sequence, such as a promoter sequence, may be maintained over the course of evolution to serve an important molecular function, such as the binding of transcription factors necessary for transcriptional regulation of that gene. To understand how AP-2 and Sp1 regulate the expression of *KiSS-1* gene, we examined and analyzed the potential regulatory regions of *KiSS-1* gene among different species (Figure 4). As sequence homology appeared to drop sharply after the first 1.2 kb, we initially focused our promoter analysis within this region, which was then cloned into a

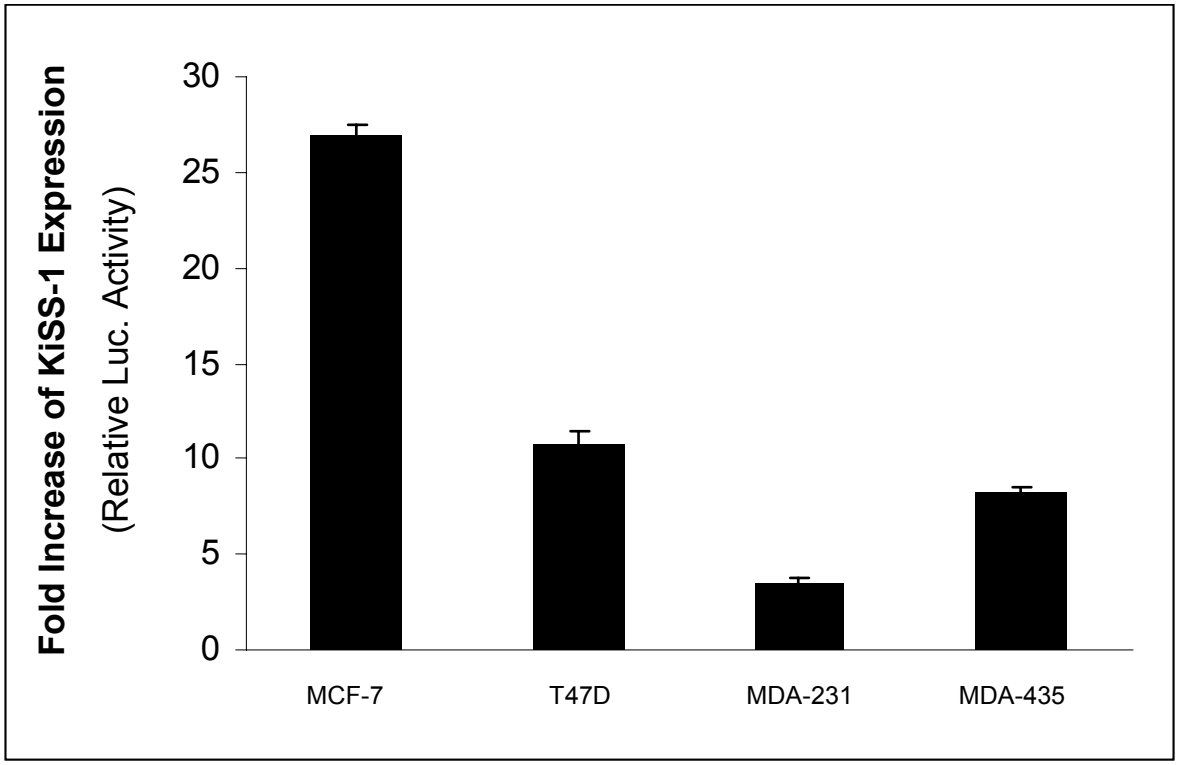

Figure 3: Co-Transfection of AP-2α and Sp1 Leads to Increased KiSS-1 Expression. Significant fold up-regulation of a KiSS-1 promoter-driven luciferase expression was observed in all breast cancer cell lines when co-transfected with both AP-2α and Sp1.

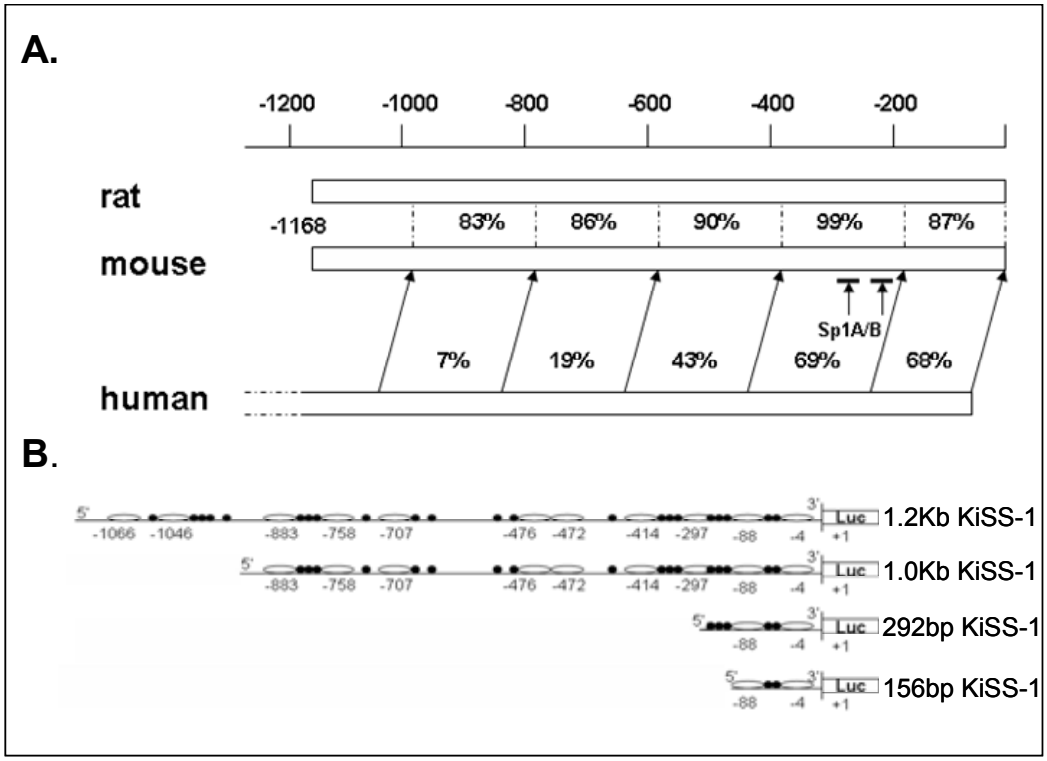

Figure 4: Identification of Regulatory Regions of KiSS-1 by AP-2α and Sp1 Transcription Factors. (A) Sequence conservation of the KiSS-1 promoter regions. Human sequences were aligned to that from different species, including chimp, dog, mouse, rat, chicken, fugu, and zebra fish. Significant similarity was observed up to nearly 1.2 Kb, but the highest homology was seen within the first 600 bp of this regulatory region. Sp1A/B indicates two conserved Sp1 sites. (B) Schematic of regulatory region of KiSS-1 depicting the potential AP-2α (unfilled ovals) and Sp1 (filled circles) transcription factor binding elements.

luciferase reporter vector. Sequence analysis of the highly conserved 400-bp region (~70% homology) located 5' proximal to the *KiSS-1* coding region contains three consensus AP-2α binding elements flanked by multiple Sp1 sites (Figure 4). Given that the *KiSS-1* promoter, much like other AP-2-regulated genes, contains multiple AP-2αbinding elements and a regulatory region rich in GC-content, it became a likely candidate gene regulated by AP-2/Sp1 complexes in tumor cells.

AP-2α and Sp1 Synergistically Activate KiSS-1 Transcription

To determine whether AP-2 and Sp1 were involved in transcriptional regulation of the KiSS-1 tumor metastasis suppressor gene, luciferase reporter constructs bearing 1.2-kb region of the *KiSS-1* promoter were transfected into breast cell lines along with AP-2, Sp1, or both. Using β-galactosidase to normalize for transfection efficiencies, we analyzed the luciferase activity driven by the *KiSS-1* promoter. Results showed that while KiSS-1-luciferase activity was only slightly increased upon overexpression of either AP-2 or Sp1 in MCF-7 cells, *KiSS-1* promoter activity was increased more than 10-fold in cells co-transfected with both AP-2 and Sp1 constructs (Figure 5). Additionally, luciferase constructs bearing serial deletions of the *KiSS-1* promoter were assayed for activation upon co-expression of AP-2α and Sp1 to determine which regions of the *KiSS-1* promoter modulated transcriptional activation by AP-2α and Sp1. As shown in Figure 5, the 292-bp KiSS-1 promoter luciferase construct was synergistically activated by co-transfection of AP-2α and Sp1 similar to results using the 1.2-kb and 1.0-kb luciferase constructs, whereas the 156-bp *KiSS-1* promoter construct showed significantly less activation (Figure 5), suggesting that the 292-bp promoter contains the regulatory elements essential for

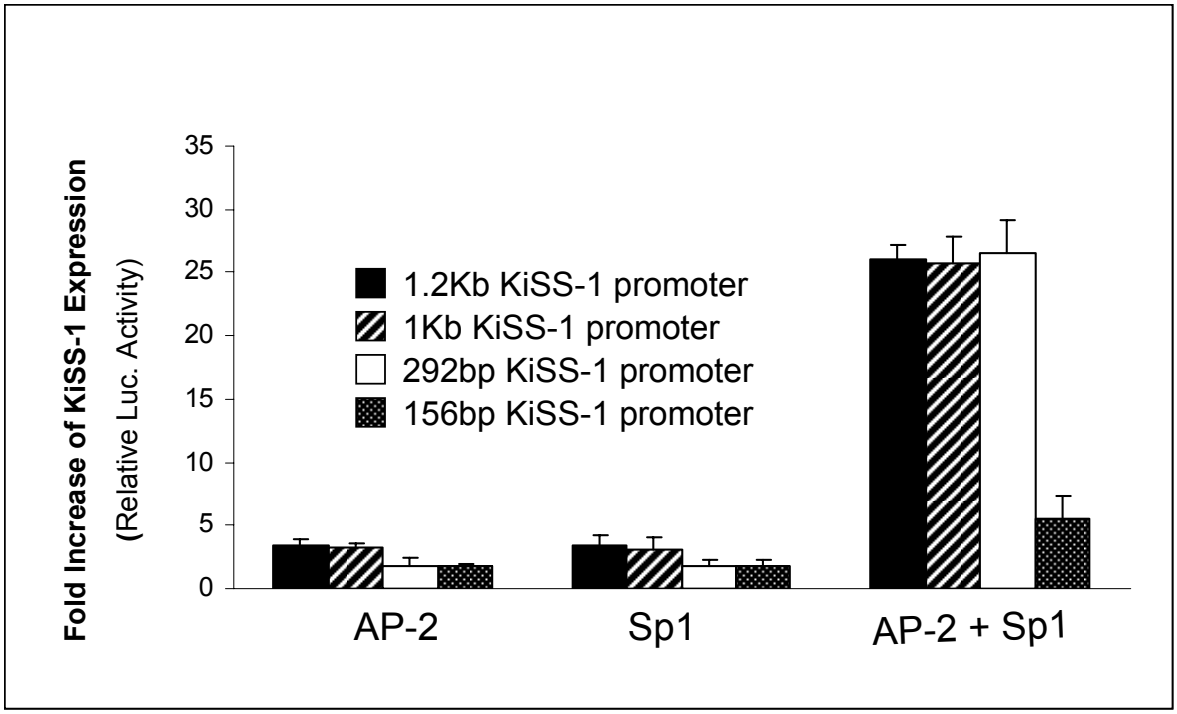

Figure 5: Co-Transfecton of AP-2α and Sp1 Leads to Increased KiSS-1 Promoter Activation. Activation of different KiSS-1 promoter-derived luciferase constructs by AP-2α and Sp1 transcription factors. Luciferase assays using serial truncation of KiSS-1 promoter demonstrate that the region modulated by Sp1 and AP-2α lies within -292 bp of the sequence, as this AP-2α modulated transcriptional activation is ablated when assaying shorter regions of the KiSS-1 promoter sequence.

transcriptional regulation by the AP-2α and Sp1 complex and that the promoter region sensitive to AP-2α and Sp1 control intervenes between these sites (156 and 292-bp).

AP-2α-activated KiSS-1 Transcription Is Independent of Direct Promoter Binding But through Interaction with Sp1 at Two Consensus Sp1 Sites

To understand the molecular mechanism of *KiSS-1* gene transcriptional regulation by AP-2α and Sp1, we examined whether direct DNA binding of AP-2α and Sp1 is essential for the remarkable activation of *KiSS-1* promoter. AP-2 and Sp1 constructs lacking the DNA-binding domains of each transcription factor (AP-2B and Sp1-ΔDBD) were co-transfected into MDA-435 and MCF-7 cells with the 292-bp KiSS-1 promoter luciferase construct and measured for luciferase activity (Figure 6). As expected, dominant negative Sp1-ΔDBD failed to activate KiSS-1 promoter-mediated luciferase activity when co-transfected with AP-2α. However, AP-2B, the truncated AP-2α lacking the C-terminal DNA-binding domain, significantly increased luciferase activity to the same degree as the wild-type form of AP-2 when co-transfected with Sp1 transcription factor. These results suggest that the DNA-binding domain of AP-2α is not necessary in modulating KiSS-1 transcriptional activity in human breast cancer cells. To determine the discrete transcriptional binding sites essential in mediating AP-2α/Sp1 transcriptional regulation of KiSS-1, we generated additional truncation mutants of 150, 190, 230, 260, and 300-bp in the luciferase reporter plasmid, as shown in Figure 7. These truncated reporter plasmids were co-transfected with AP-2α and Sp1 in breast cell lines for further luciferase analysis. As shown in Figure 7, the promoter region between +230 and +260 demonstrated the greatest sensitivity to AP-2α/Sp1 transcription factors. Co-transfection of AP-2α and Sp1

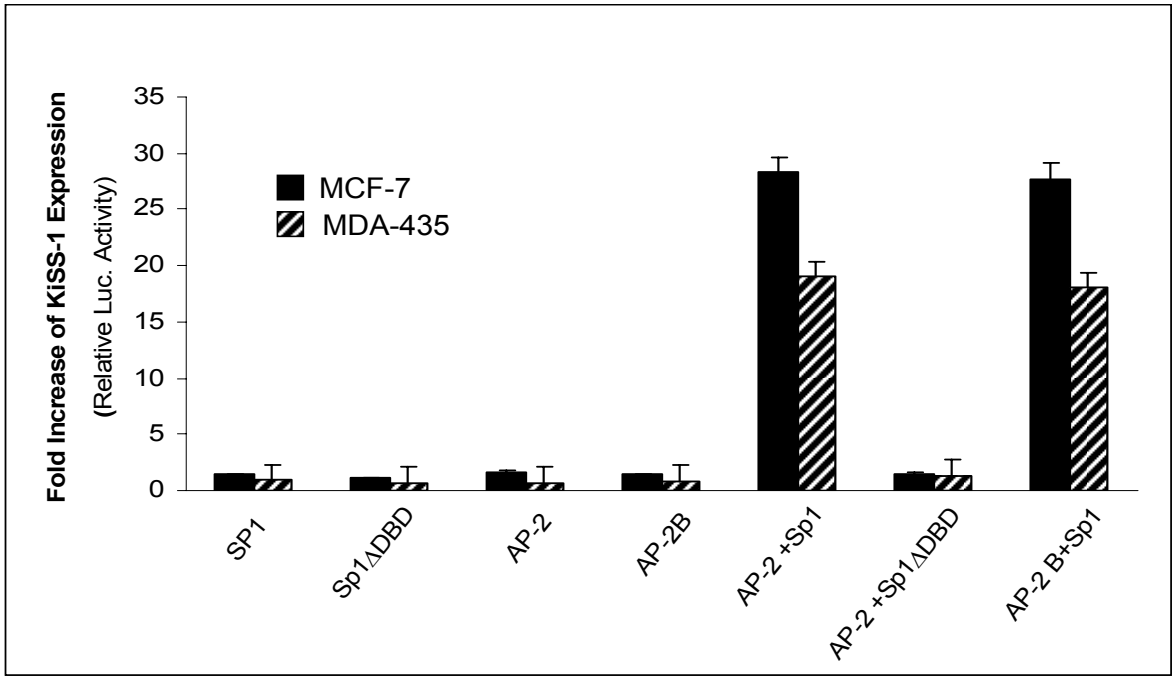

Figure 6: Activation of KiSS-1 Expression by AP-2α is Independent of Direct Promoter Binding. Activation of KiSS-1 promoter activity by wild-type AP-2α, Sp1, DNA-binding deficient mutants AP-2α (AP-2B) and Sp1 (Sp1ΔDBD) using luciferase assays. AP-2α and Sp1 synergistically enhances KiSS-1 activation, as does the C-terminal deletion mutant of AP-2 (AP-2B) when co-transfected into MDA-435 and MCF-7 breast cell line, while co-transfection of the DNA-binding domain deletion mutant of Sp1 (Sp1ΔDBD) failed to activate the KiSS-1 promoter.

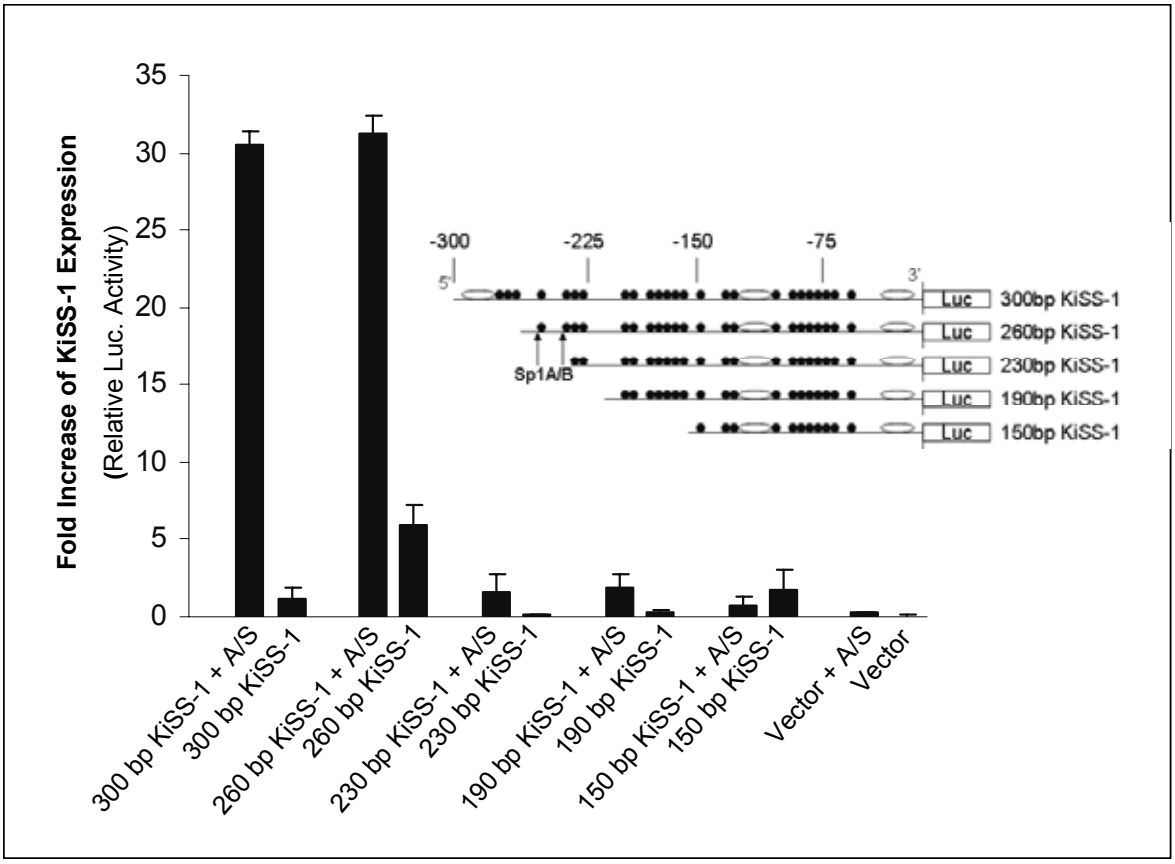

Figure 7: Promoter Region Between +260 and +230 Modulates AP-2α and Sp1 Transactivation. Additional serial deletions of the KiSS-1 promoter of -300, -260, -230, -190, and -150 bp in length were assayed in luciferase experiments, demonstrating that the region most notably modulated by Sp1 and AP-2α lies between -230 and -260 bp which consists of two highly conserved Sp1 putative binding sites (Sp1-A and Sp1-B).

significantly stimulates the activity of the 260-bp KiSS-1-Luc, similar to that exhibited by the 300-bp KiSS-1-Luc construct, but did not contain any putative AP-2 sites. The 260-bp KiSS-1-Luc does, however, contain two consensus Sp1 sites in tandem, identified as Sp1A and Sp1B (Figure 7). To further determine if these two Sp1 sites were essential in mediating the transcriptional modulation of the KiSS-1 promoter by AP-2α and Sp1, constructs of single targeted deletion (mutant Sp1-A or mutant Sp1-B) at these two sites as well as double deletions (mutant Sp1-A/B) at both sites of the *KiSS-1* promoter were generated and tested in luciferase assay (Figure 8). Results showed that mutation at either Sp1 site rendered the *KiSS-1* promoter insensitive to the regulation of AP-2α and Sp1 transcription factors in MCF-7 cells (Figure 8), suggesting that the two Sp1 sites are essential for the observed regulation of *KiSS-1* promoter by AP-2α and Sp1 transcription factors.

Sp1 and AP-2α Form a Complex at the Sp1 Sites of KiSS-1 Promoter

To determine the complex formation of Sp1 and Ap-2α at the two Sp1 sites in mediating AP-2α/Sp1 regulation of the *KiSS-1* gene transcription, gel mobility shift analysis (EMSA) was performed using the GC-rich sequence spanning +230 to +260 of the *KiSS-1* promoter (Figure 9). Results of EMSA using AP-2α and Sp1-transfected MCF-7 cells showed that both Sp1 and Sp3 bind to the 30-bp γ-^{32}P-labeled probe (*lanes 2–4*), however this DNA-protein interaction was abrogated in the presence of unlabeled competitor (*lane 5*). The negative control showing labeled probe in the absence of nuclear extract is shown in *lane 1*. Upon addition of a Sp1-specific antibody, the migration of the DNA-protein complex was sterically hindered by the bound antibody, resulting in a

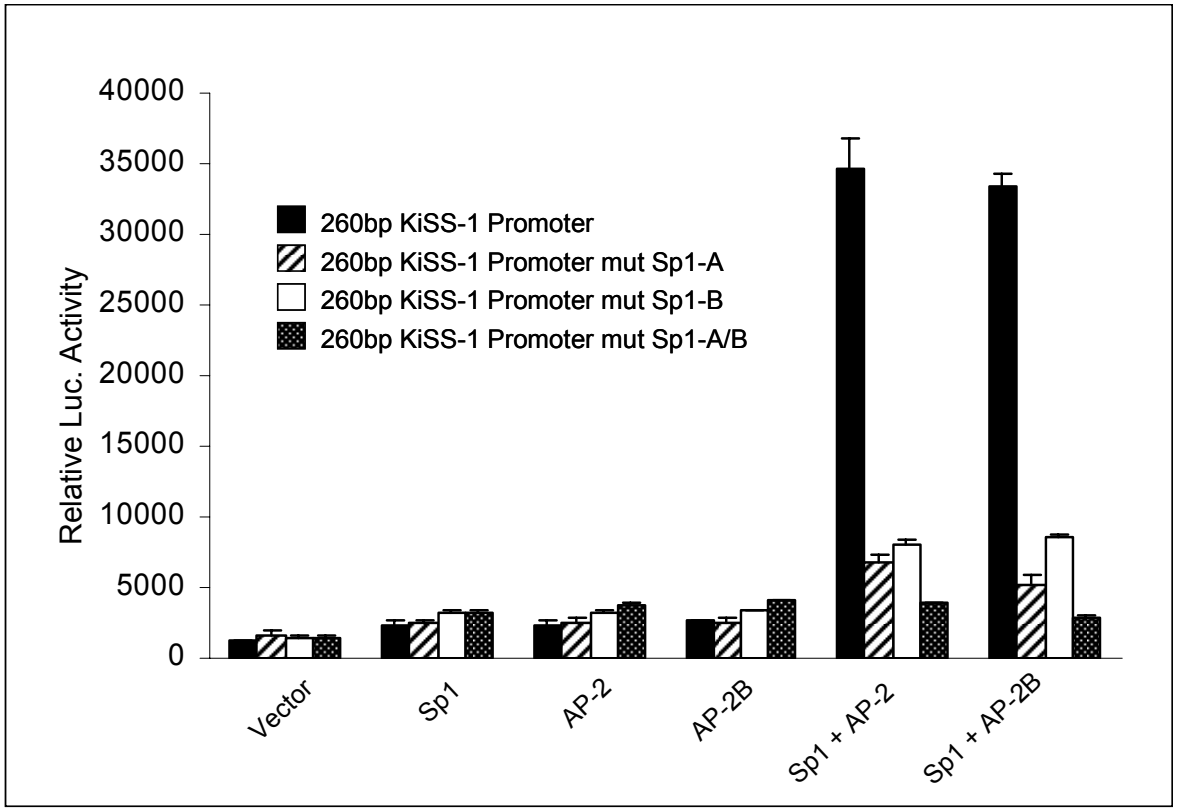

Figure 8: Targeted Mutation of Two Consecutive Sp1 Sites Leads to AP-2α/Sp1 Promoter Insensitivity. Targeted mutations of the two Sp1 sites illustrated that both sites (Sp1-A and Sp1-B) were essential in mediating the AP-2α/Sp1-modulated transactivation of the KiSS-1 promoter.

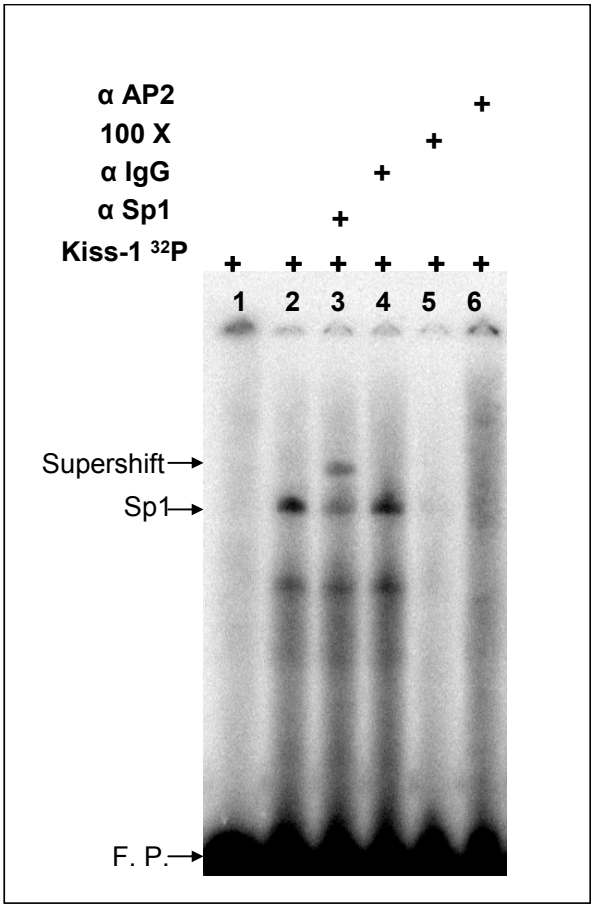

Figure 9: EMSA Analysis Shows Sp1 Binding at KiSS-1 Promoter. EMSA assay using cells transfected with AP-2α and Sp1, demonstrates two Sp1 binding elements (Sp1A and Sp1B) are binding sites for Sp1/AP-2 complexes. This 30bp sequence was used in EMSA, and lane 3 indicated Sp1-specific antibody supershifted probe, whereas the Ig-G negative control showed no shift (lane 4). Additionally, bands resulting from Sp1-DNA complex are greatly reduced upon addition of unlabeled 100X competitor (lane 5). Incubation of anti-AP-2 antibody results in loss of SP1-DNA complexes indicating a competition for binding (lane 6).

supershifted third band (*lane 3*). However, the ability to supershift the labeled probe was specific for the Sp1 antibody, because the antibody for IgG failed to exhibit any ability to complex with this protein-bound probe (negative control, *lane 4*). Incubation of the labeled promoter fragment with anti-AP-2 antibody resulted in a diffusion of the bands formed by the Sp1-DNA complex, indicating that AP-2 may also bind to this complex. To further demonstrate that AP-2α exists in the Sp1-DNA complex, we performed chromatin immunoprecipitation (ChIP) assays using sheared DNA isolated from AP-2α/Sp1 co-transfected MCF-7 cells. Overnight immunoprecipitation of chromatin-bound DNA using antibodies to Sp1, AP-2, and IgG was followed by PCR using primers that amplified the 100-bp region spanning the length of the two Sp1 sites (Sp1-A and Sp1-B) (Figure 10A). As shown in the ChIP assays (Figure 10B), anti-Sp1 and anti-AP-2 antibodies were capable of immunoprecipitating the KiSS-1 promoter fragment containing the two Sp1 sites (*lanes 4* and *5*), however, immunoprecipitation using anti-IgG failed to produce a PCR product (Figure 10B, *lane 3*).

AP-2α Interacts Directly with Sp1 at an N-terminal Domain of the Protein

Our data indicate that both AP-2α and AP-2α C-terminal deletion mutant (AP-2B) lacking the DNA-binding domain synergistically enhance Sp1-mediated activation of KiSS-1, suggesting that the effect of AP-2α is through the interaction with Sp1, independent of its ability to directly bind the AP-2 consensus sites of the *KiSS-1* promoter.

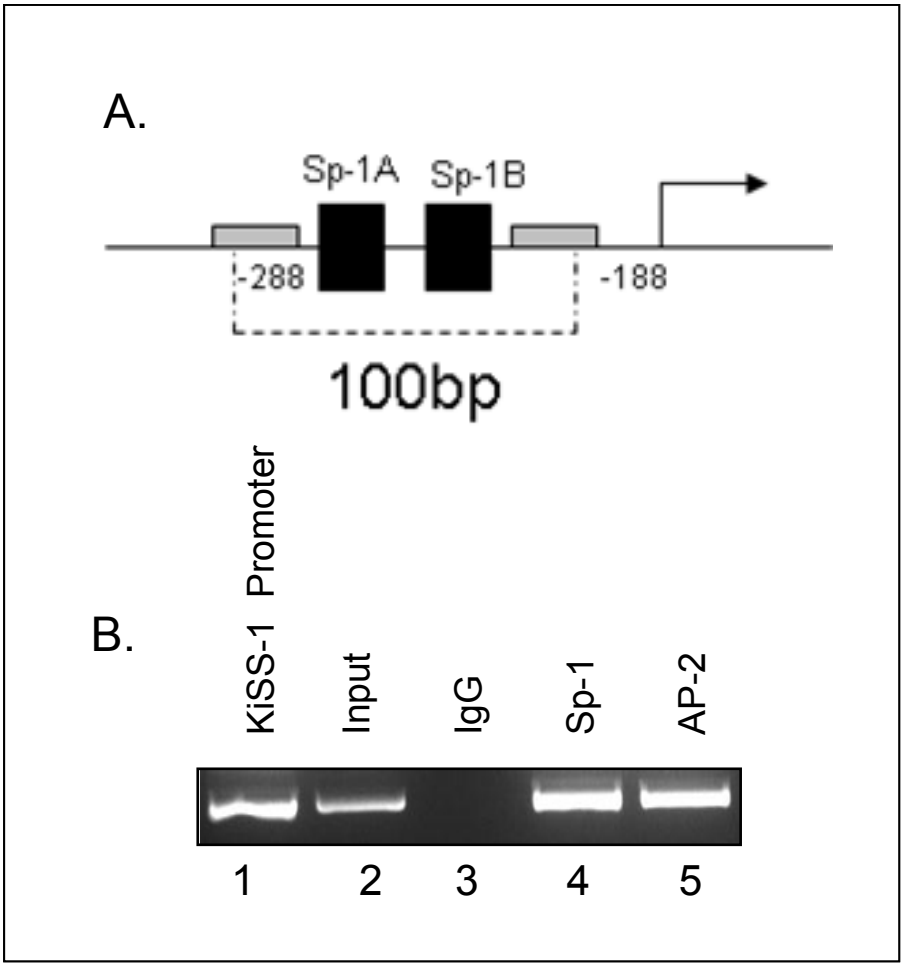

Figure 10: Chromatin Immunoprecipitation Shows Binding of Sp1 and AP-2α to KiSS-1 Promoter. (A) Schematic of the KiSS-1 promoter region containing two Sp1 binding elements (Sp-1A and Sp-1B) analyzed by ChIP assay. PCR primers (gray bars) amplified a 100 bp region ranging from-188 to -288. (B) ChIP using MCF-7 cell lysates transfected with AP-2α and Sp1. Chromatin was immunoprecipitated using antibodies to Sp1 and AP-2 (lane 4 and lane 5). Anti-IgG was used as a negative control (lane 3). The 1.2 Kb KiSS-1 promoter was used as a positive control for the PCR (lane1).

To demonstrate the N-terminal region of AP-2α without the C-terminal DNA-binding domain directly interacts with Sp1 in the cell, MCF-7 breast cancer cells were co-transfected with Sp1, AP-2, and AP-2B or control vector. Cell lysates were incubated with immunoblotted for the alternate antibody (Figure 11). Immunoprecipitation of cell lysates that overexpress AP-2α or AP-2B by anti-AP-2 antibody demonstrated the presence of Sp1 protein in the immunocomplexes (Figure 11A, *lanes 2* and *3*) as compared with the vector control (*lane1*). Conversely, both AP-2α and AP-2B were immunoprecipitated with the Sp1 antibody (Figure 11B, *lanes 2* and *3*) in cells that overexpressed either protein, whereas vector-transfected cell lysate did not show any immunoreactivity (*lane 1*). Relative amounts of AP-2α and Sp1 levels are also shown (Figure 11, *C* and *D*). These results demonstrate the direct interaction of the N-terminal region of AP-2α with the Sp1 transcription factor in breast cell lysate.

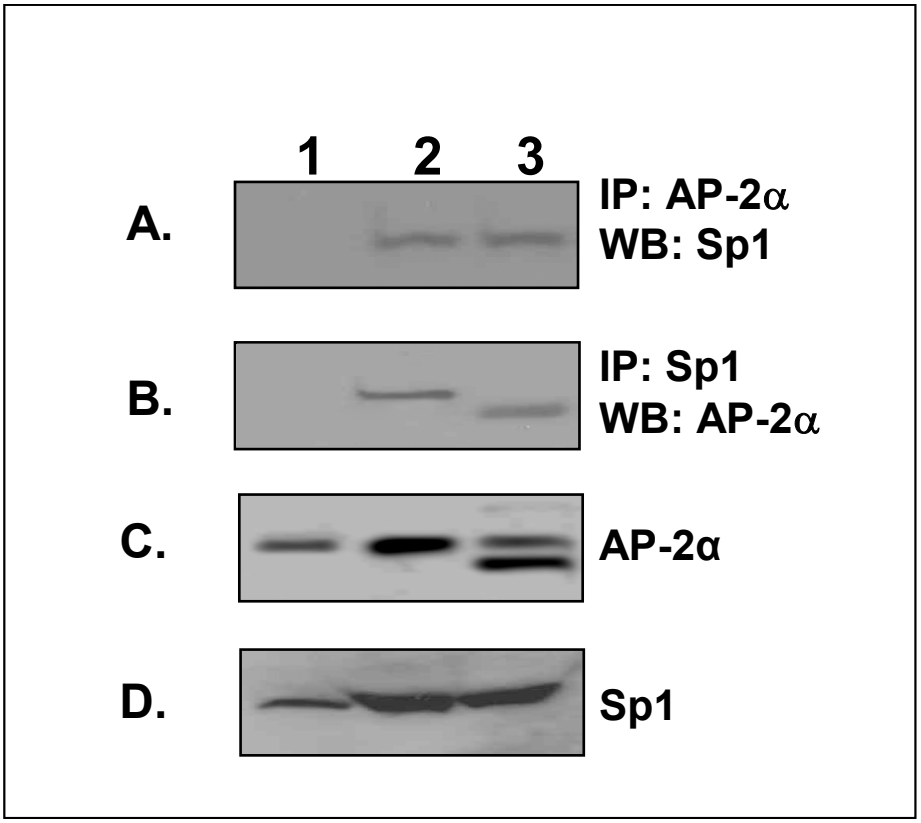

Figure 11: Direct Interaction of AP-2α with Sp1 Transcription Factor. (A) Co-immunoprecipitations of Sp1 with wild-type AP-2α and mutant AP-2B using the N-terminal AP-2α specific antibody in lysates expressing AP-2α (lane 2), AP-2B (lane 3) and vector (lane 1). The protein complexes were immunoblotted with Sp1 antibody. (B) Association of AP-2α and DNA-binding domain mutant (AP-2B) with Sp1 in transfected MCF-7 cells. Stably transfected MCF-7 cells expressing AP-2α (lane 2), AP-2B (lane 3), and vector only (lane 1) were co-immunoprecipitated with specific Sp1 antibody and immunoblotted with antibody against the N-terminal domain of AP-2α. Western blot analysis showing AP-2 (C) and Sp1 (D) expression in transfected MCF-7 lysates used in co-immunoprecipitation assays.

Discussion

Treatment of metastatic breast cancer cells with synthetic KiSS-1 peptide as well as ectopic overexpression of KiSS-1 in these cells has been shown to significantly alter overall cellular morphology and behavior, thus hindering metastasis (Lee and Welch, 1997a; Ohtaki et al., 2001; Muir et al., 2001; Kotani et al., 2001). The expression of KiSS-1, like other tumor suppressors, is commonly reduced or completely ablated in a variety of different cancers (Masui et al., 2004; Lee and Welch, 1997b; Ikeguchi et al., 2004; Sanchez-Carbayo et al., 2003). As the level of KiSS-1 expression has been correlated to patient survival and severity of metastatic development, KiSS-1 has become an effective biomarker for the metastasis of these cancers (Sanchez-Carbayo et al., 2003). In this study, we demonstrate that the expression of KiSS-1 is directly correlated with the expression level of another known tumor-suppressor, the AP-2α transcription factor. Highly metastatic breast cell lines exhibit little to no AP-2α expression, paralleling the loss of the *KiSS-1* gene in these cells. Additionally, we found that AP-2α and Sp1 synergistically activate the transcriptional activation of *KiSS-1* promoter in multiple breast cancer cell lines via direct interaction of these two transcription factor at two tandem Sp1-binding sites of the promoter.

Although MCF-7 exhibited higher levels of endogenous AP-2α and Sp1 expression relative to the other breast lines (Figure 2), the significant increase in *KiSS-1* promoter activation seen upon transfection of both transcription factors (Figure 3) is likely due to the additive effect caused by the overexpression of these proteins in the same cells transfected with the KiSS-1 promoter construct. The *KiSS-1* promoter was found to contain multiple

AP-2α-binding sites, all of which were flanked by clusters of Sp1 sites; furthermore, this region was rich in G-C content, making KiSS-1 a likely candidate for regulation by AP-2α and Sp1 complex. Serial truncations of the KiSS-1 regulatory region demonstrated that the AP-2α/Sp1 regulation was mediated through a region of the promoter between +230 and +260-bp with only two putative Sp1 consensus sites. The ability of Sp1 to bind to this discrete 30-bp sequence was confirmed in EMSAs in which an Sp1-specific antibody could bind this protein-bound promoter segment as evidenced by its ability to supershift the complex. EMSA results for anti-AP-2α-antibody incubated nuclear extracts showed a diffusion of the bands formed by the Sp1-DNA complex, thus indicating that AP-2α may also bind this complex. Additional chromatin immunoprecipitation using the region of the promoter carrying the two tandem Sp1 sites demonstrated that both AP-2α and Sp1 were capable of promoter interaction. Mutation analysis at these two putative Sp1 sites further demonstrated the binding of the AP-2α/Sp1 complex at these two sites and the essential of these two sites in AP-2- and Sp1-mediated transcriptional activation of KiSS-1. Although AP-2α is necessary to mediate transcriptional regulation of the *KiSS-1* promoter, our EMSA results, luciferase-reporter assays using a dominant negative form of AP-2α that lacks the C-terminus DNA-binding domain, and the co-immunoprecipitation assays demonstrated that the N-terminal domain of AP-2α forms a protein complex to regulate transcriptional activation of KiSS-1 and that the role of AP-2α is independent of its promoter-binding ability. Taken together, our data suggest a possible model for transcriptional activation of KiSS-1 in breast cells (Figure 12). In normal cells, in which AP-2α is regularly expressed, the N-terminal region of AP-2α interacts with Sp1, which

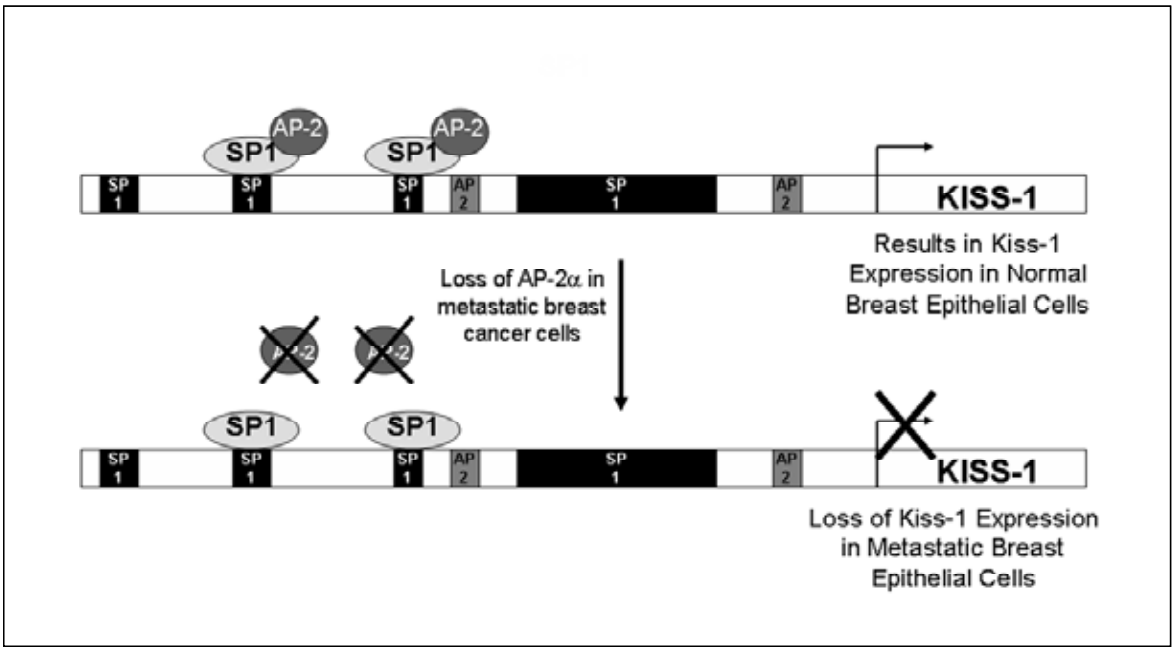

Figure 12: Proposed Model for the Regulatory Mechanism of KiSS-1 Expression in Both Normal and Metastatic Cancer. Proposed model for the regulatory mechanism of KiSS-1 expression in both normal and metastatic breast cancer. Our data supports that when AP-2α is regularly expressed as it is in normal breast tissues, the N-terminal region of AP-2α directly interacts with Sp1 to transactivate the KiSS-1 promoter and KiSS-1 expression. However, loss or reduced expression of AP-2α leads to the disruption of the AP-2α/Sp1 transcriptional complexes, and consequently, the loss or reduced expression of KiSS-1 suppressor gene in metastatic cancer cells.

binds to the two-consensus Sp1-binding sites. The AP-2α and Sp1 complex initiates transactivation of the *KiSS-1* promoter and lead to the expression of *KiSS-1* gene (Figure 12). Alternatively, when AP-2α expression is lost, as commonly found in many cancer types, depletion of AP-2α/Sp1 transcription factor complex leads to a loss of KiSS-1 transcriptional activation (Figure 12). Contrary to conventional transcriptional regulation by AP-2α, in which AP-2α recognizes and binds to its consensus DNA-binding site 5'-GCCCNNNGGC-3' (Williams and Tjian, 1991), our results demonstrate a novel mechanism in which AP-2α regulation is mediated through two tandem Sp1 elements located between +230 to +260-bp and requires direct protein-protein interaction of the N-terminal portion of AP-2α with Sp1 protein. KiSS-1 was originally identified to reduce melanoma metastasis by 95% using microcell-mediated transfer of chromosome 6 (Lee and Welch, 1997a). Although KiSS-1 was later accurately mapped to chromosome 1q32-q41, it was believed that this region of the chromosome 6 contained a key, and as yet, unidentified KiSS-1 regulator that activated KiSS-1 expression upon transfer into melanoma cells. Interestingly, AP-2α is located in the short arm of chromosome 6, the exact region believed

to regulate KiSS-1; therefore, AP-2α may in fact be the missing KiSS-1 regulatory factor. Our data strongly establish a correlation between the loss of the AP-2α transcription factor and loss of the KiSS-1 metastasis suppressor in breast cancer cell lines. Loss of AP-2α has been implicated in the development of many other cancers besides invasive breast carcinomas, including colorectal, prostate, and ovarian cancer, renal cell carcinoma, and melanoma. Because KiSS-1 expression has also been found to be reduced in many of these same cancers, it is possible that KiSS-1 expression in these tissues is similarly reliant on the interaction of AP-2α with Sp1 and that the loss of AP-2α is paralleled by the loss of KiSS-1 expression, causing such cells to effectively lose another barrier to metastatic development. For this reason, our current studies are extending these observations of the role of AP-2α in mediating KiSS-1 expression in other cancers to identify similar mechanisms underlying the loss of KiSS-1 expression in such tissues and cancers.

CHAPTER IV

REGULATION OF KISS-1 METASTASIS SUPPRESSOR GENE EXPRESSION IN MELANOMA BY SPECIFICITY PROTEIN-1 AND COACTIVATOR DRIP-130

Overview

The loss of the metastasis suppressor gene, KiSS-1 or KiSS1, has been strongly correlated to the progression of metastases in numerous types of cancers. The mechanism through which KiSS-1 is lost during metastasis, however, is still not completely known. Previous studies have shown that genetic material on human chromosome 6q16.3-q23 is essential for KiSS-1 expression in normal tissues. Additionally, microcell-mediated transfer of this chromosome in cancerous tissue results in rescued expression of KiSS-1 and reduced metastatic phenotype. Here, we show that loss of Sp1-coactivator protein DRIP-130, which is encoded by human chromosome 6q16.3-q23, results in reduced KiSS-1 promoter activation in highly malignant melanoma cells. Co-expression of Sp1 and DRIP-130 not only rescues KiSS-1 expression, but also induces an inhibition of the invasive and migratory behavior in highly metastatic melanoma cells, similar to the overexpression of KiSS-1 metastasis suppressor gene in those cells. Furthermore, we demonstrate that KiSS-1 expression is regulated by Sp1 elements within the first 100-bp region of the KiSS-1 promoter and that targeted deletion of a single GC-rich region spanning +93 to +58 interrupts Sp1- and DRIP-130-modulated transcriptional control of KiSS-1 expression. Our results thus suggest that DRIP-130 is a key regulator in KiSS-1 transactivation in

normal tissue, and that the loss of DRIP-130 expression, as a result of the gross loss of human chromosome 6q16.3-q23, provokes increased tumor metastasis.

Introduction

Metastasis is an involved process in which cancer cells spread through the circulatory system, adhere and invade new tissues and then develop as secondary cancers (Pantel and Brakenhoff, 2004). In the past decade, several genes have been identified which interfere in the metastatic cascade, thus inhibiting cancer metastasis; such genes are consequently called metastasis suppressors (Steeg et al., 2003). As these genes were only recently identified, the mechanisms by which they inhibit the progression and metastasis of cancers largely remain a mystery, as does the regulation of their expression in both normal and tumorigenic tissues. KiSS-1, or KiSS1, is a precursor for secreted peptide ligands for a G-protein coupled receptor, named hGPR54 (Kotani et al., 2001), or hOT7T175 (Ohtaki et al., 2001), or AXOR12 (Muir et al., 2001) or KiSS1 receptor (Stafford et al., 2002). The active peptide ligands are designated as Metastin (Ohtaki et al., 2001) or Kisspeptins (Kotani et al., 2001). Although KiSS1 and its G-protein coupled receptor have been shown to suppress tumor metastasis in different tumors, including breast tumor, lung tumor, and melanoma (Lee and Welch, 1997a; Lee and Welch, 1997b), the mechanism for KiSS-1 suppression is still unknown. Recent studies suggest that KiSS-1 activates G-protein mediated PLC-Ca^{2+} signaling pathways in different cell types as well as ERK and p38 MAPK pathways to inhibit cell mobility, migration, and invasion *in vitro* (Ohtaki et al., 2001; Stafford et al., 2002; Kotani et al., 2001; Muir et al., 2001; Yan et al., 2001).

Microcell-mediated transfer of highly metastatic melanoma was initially used to identify KiSS-1 as a metastasis suppressor gene, as it was shown to reduce invasive and migratory properties without affecting tumorigenicity (Lee and Welch, 1997b). Similar to that of other known metastasis suppressors, the expression of KiSS-1 is commonly found to be inversely correlated to the degree of cancer metastasis. Since its identification, loss of KiSS-1 expression has been shown to be a good indicator for the progression of several types of cancers, including breast, melanoma, pancreatic, bladder, and esophageal squamous cell carcinoma (Masui et al., 2004; Lee and Welch, 1997a; Ikeguchi et al., 2004; Sanchez-Carbayo et al., 2003). Additionally, treatment with synthesized KiSS-1 peptide, as well as the induced overexpression of KiSS-1 *in vitro*, effectively reduces invasive and migratory properties of cancer cells (Lee and Welch, 1997a; Masui et al., 2004; Ohtaki et al., 2001; Stafford et al., 2002; Lee and Welch, 1997b). As previously published, the KiSS-1 promoter is highly GC-rich and computational analysis of this region revealed multiple Sp1 sites, which our lab has shown to be necessary, along with the transcription factor AP-2α, to promote KiSS-1 transcriptional activation in breast cancer (Mitchell et al., 2006). Our earlier data supported a model by which the loss of AP-2α which accompanies the progression of multiple forms of cancer resulted in the overall loss of KiSS-1 expression in breast cancer cells and the loss of the metastatic inhibition mediated by KiSS-1 (Mitchell et al., 2006). Recent evidence suggests the possible involvement of KiSS-1 regulatory genes in chromosome 6 (6q16.3-q23) in tumor metastasis suppression (Miele et al., 2000; Shirasaki et al., 2001). Deletion of 6q16.3-q23 and loss of heterozygosity in that region correlated with decreased KiSS-1 expression, suggesting that KiSS-1 is regulated by

a possible regulatory factor in that region of the chromosome (Miele et al., 2000; Shirasaki et al., 2001). One of the major objectives of our current study is to identify the upstream regulator(s) of KiSS-1 which are encoded by chromosome 6q16.3-q23. DRIP-130, like AP-2α, is localized to the short arm of human chromosome 6, the region believed to encode the regulatory factor(s) of KiSS-1 (Goldberg et al., 2003). DRIP-130, also known as CRSP130, is one of 15 subunits which compose the transcriptional cofactor complex CRSP/Mediator, which is required for transcriptional activation by Sp1 (Ryu et al., 1999; Ryu and Tjian, 1999; Taatjes et al., 2004). It has also been suggested that DRIP-130 itself acts as a metastasis suppressor, as its expression in clinical samples of melanomas is directly correlated with other metastasis suppressors as detected by quantitative real-time reverse-transcription PCR (Goldberg et al., 2003).

In this study, we provide data to support a mechanism by which Sp1 strongly regulates transcriptional activation of the KiSS-1 promoter and KiSS-1 gene expression by interacting with the DRIP-130 subunit of the CRSP cofactor complex. In human metastatic melanomas, the loss of the region in chromosome 6 (6q16.3-q23), which encodes the DRIP-130 subunit of the CRSP coactivator complex, results in the consequent loss of KiSS-1 metastasis suppressor gene expression. Additionally, we report that re-introduction of DRIP-130 in highly metastatic melanoma cells induces higher KiSS-1 expression, and that coexpression of Sp1 and DRIP-130 decreases the invasive and migratory properties of the metastatic melanoma cells. Furthermore, we demonstrate that KiSS-1 expression is modulated by Sp1 elements within the first 100-bp region of the KiSS-1 promoter and that targeted deletion of a single GC-rich region spanning +93 to +58 of the promoter interrupts

Sp1- and DRIP-130-modulated transcriptional control of KiSS-1 expression. Therefore, our data provides a regulatory mechanism for the loss of *KiSS-1* gene expression commonly seen in metastatic melanomas and correlates it with the gross loss of genetic material on human chromosome 6q16.3-q23 which accompanies this loss and leads to heightened tumor metastasis.

Identification of the Regulatory Region of KiSS-1 Promoter by Sp1 and its Coactivator DRIP-130 in Melanoma

The full-length human KiSS-1 promoter has a high GC-content and computer analysis has indicated the presence of multiple Sp1 sites throughout the sequence. One of the most GC-rich regions of the KiSS-1 promoter, containing more than 10 overlapping putative Sp1 sites, is found within the first hundred bases of the KiSS-1 start site (see Figure 13A). Recent evidence has revealed that the transcription factor, Sp1, requires the presence of other cofactors for proper assembly on Sp1-modulated promoters, such as the CRSP complex (Ryu et al., 1999). Our recent study has demonstrated that Sp1, together with AP-2α, regulates KiSS-1 transcriptional activity in breast cancer cells (Mitchell et al., 2006). To determine whether the transcriptional effect of Sp1 on KiSS-1 expression in melanoma is modulated by the Sp1 co-activator, DRIP-130, we examined the effects of Sp1 and DRIP-130 on the transcriptional activation of KiSS-1 promoter using a series of KiSS-1 promoter regions subcloned into the luciferase reporter gene constructs (see Figure 13A). Luciferase assays were carried out as previously described and normalized using β-galactosidase levels (Mitchell et al., 2006). As shown in Fig. 13B, whereas single transfections of DRIP-130 and Sp1 had no effect on activation of the first 1Kb region of the

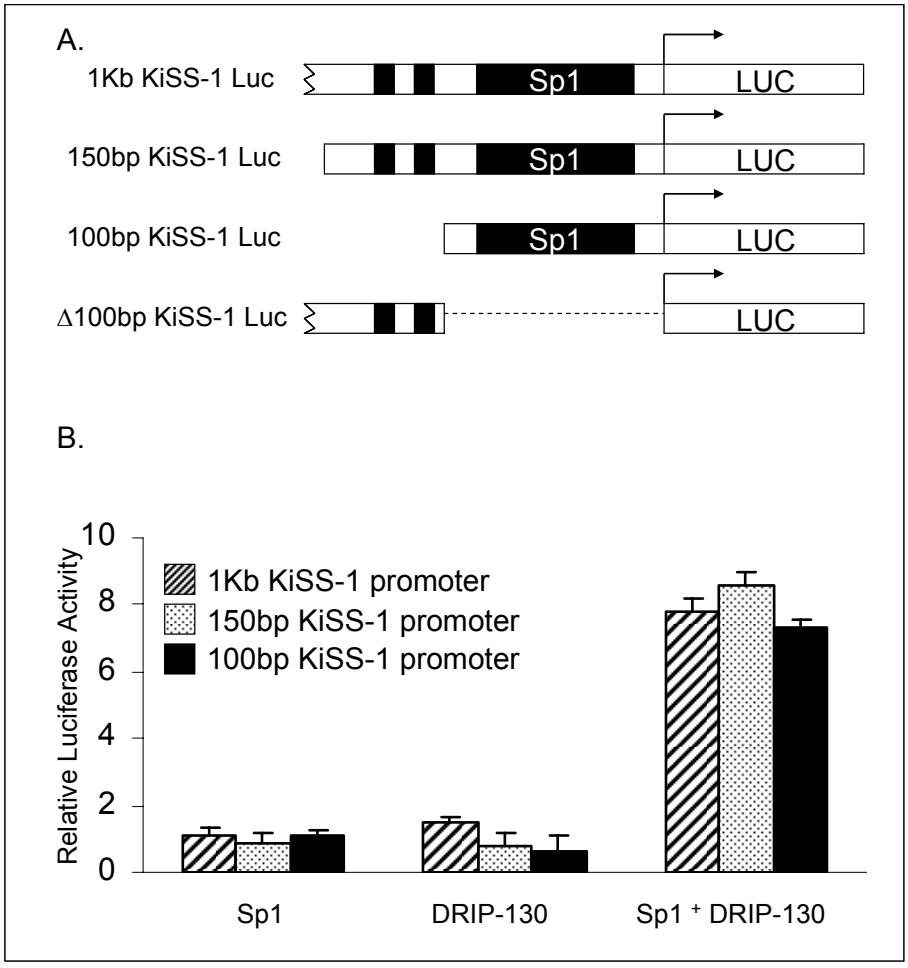

Figure 13: The KiSS-1 Promoter Contains Multiple Overlapping Sp1 Sites Regulated by DRIP-130 and Sp1. (A) Schematic representation of human KiSS-1 promoter-driver luciferase constructs containing 35bp cluster of overlapping putative Sp1 transcription factor binding elements (SP1 CLUSTER) in addition to two smaller individual putative Sp1 sites (black boxes). (B) Sp1 and DRIP-130 activate different KiSS-1 promoter-luciferase constructs, including the 1Kb, the 150bp, and the first 100 base pairs preceding the start site.

KiSS-1 promoter, co-expression of both Sp1 and DRIP-130 resulted in a 6-8 fold increase in the highly metastatic melanoma cell line, WM2664 (Fig. 13B). In an effort to determine the discrete region modulating the Sp1 and DRIP-130 response, luciferase assays using serial truncations of the KiSS-1 promoter sequence were performed. Nearly the same fold increase in KiSS-1 transactivation could be observed using the 1000-, 150- and the 100-base pair sequence of the KiSS-1 promoter, suggesting that the region modulating KiSS-1 transcriptional activation was located near the 100bp start site of KiSS-1 gene (Fig. 13B).

To examine whether the effects of Sp1 and DRIP-130 on the activation of KiSS-1 promoter are tissue/cell specific, we further measured the transcriptional activation of KiSS-1 promoter by Sp1 and DRIP-130 in four different metastatic cancer cell lines, two melanoma (WM2664 and A375SM) and two breast cancer cell lines (T47D and MDA-435). As shown Figure 14C, cotransfection of Sp1 and DRIP-130 significantly activated the luciferase activity of the KiSS-1 promoter in two metastatic melanoma cells, WM2664 and A375SM. Interestingly however, only slight activation of the KiSS-1 promoter was observed upon overexpression of Sp1 and DRIP-130 in the two highly-metastatic breast cancer cell lines, T47D and MDA-435 (Fig. 14), suggesting that the transcriptional activation of KiSS-1 by Sp1 and DRIP-130 may be tissue-specific, being regulated by Sp1 and DRIP-130 in the melanoma cell lines (Fig. 14).

KiSS-1 Levels in Highly Metastatic Melanoma Correlates Directly to the Expression of DRIP-130 mRNA and Protein

To determine whether a correlation existed between the expression of KiSS-1, Sp1 and DRIP-130 in melanoma, we examined the expression levels of these genes in

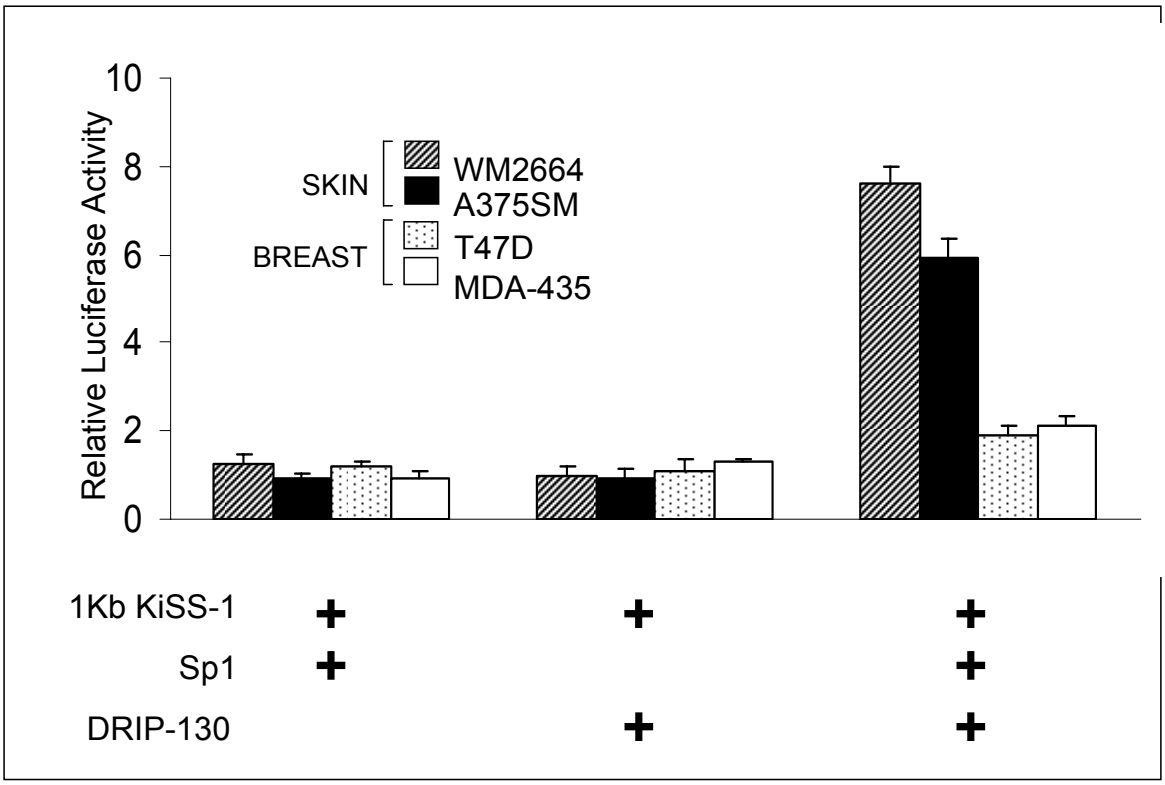

Figure 14: DRIP-130 Transcriptional Up-regulation is Cell-Type Specific. Luciferase assays in both metastatic melanoma (WM2664 and A375SM) and breast cancer cells (T47D and MDA-435) show a synergistic effect of Sp1 and DRIP-130 on transcriptional activation of the 100bp KiSS-1 promoter only in melanoma cells.

melanoma cell lines of different metastatic capacities (Figure 15). Due to the lack of a specific and effective KiSS-1 antibody, the KiSS-1 expression level in the melanoma cell lines was quantitated using RT-PCR and normalized to the β-actin level within each sample. Results showed that the more metastatic cell lines, A375SM and WM2664, showed considerably less expression of both KiSS-1 and DRIP-130, as compared with the lesser metastatic SB2 and MeWo cell lines (Fig. 15). Although a slight increase in Sp1 expression was seen in the SB2 line, Sp1 expression remained relatively constant in different cell types. Immunoblot analysis of actin expression was analyzed and used as a loading control. To determine whether reduced expression of DRIP-130 in melanoma was responsible in part for decreased KiSS-1 levels found in the more metastatic WM2664 and A375SM melanoma cell lines, DRIP-130 was singly transfected into these cells, which were then lysed and analyzed for KiSS-1 expression using RT-PCR. Since no significant increase in KiSS-1 mRNA levels was seen (results not shown), cells were then co-transfected with both Sp1 and DRIP-130 after which KiSS-1 mRNA levels were examined. Results demonstrated that co-transfection of DRIP-130 and Sp1 induced KiSS-1 expression levels in both melanoma cell lines (Fig. 16, middle, co-transfected) as compared to the untransfected cells (Fig. 16, top). Singly over-expression of Sp1 will not result in such an increase of KiSS-1 expression in either cell type. Together with our transcriptional activation assays using KiSS-1 luciferase promoters, our data suggests that over-expression of both Sp1 and DRIP-130 is required for this up-regulation of KiSS-1 in metastatic melanoma cell lines.

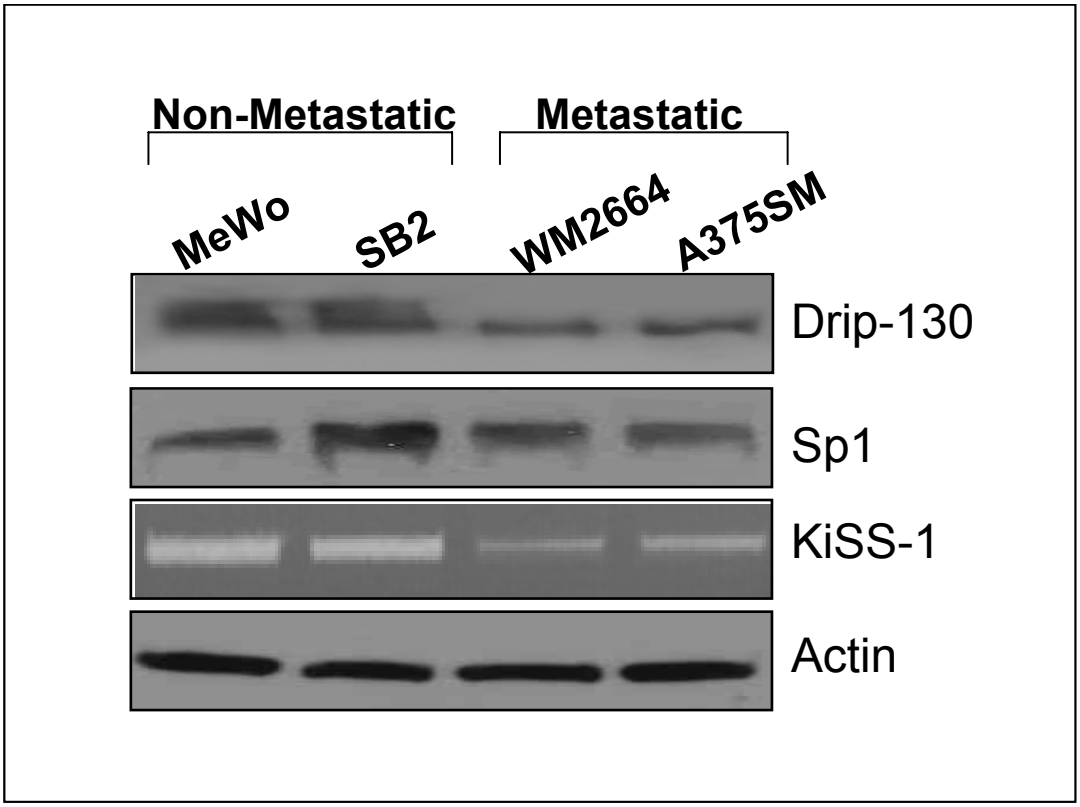

Figure 15: Reduced Expression of DRIP-130 in Highly Metastatic Melanoma Cells Correlates with the Expression Level of KiSS-1. Western blot analysis of DRIP-130 and Sp1 was compared to KiSS-1 RT-PCR analysis in both non-metastatic (MeWo and SB2) and metastatic (WM2664 and A375SM) melanoma cells. Reduced DRIP-130 expression seen in metastatic melanoma correlated directly with reduced mRNA levels of KiSS-1. Sp1 levels remained relatively unchanged between cell lines examined. Actin controls for Western analysis and β-actin controls for RT-PCR analysis (data not shown) were carried out to ensure equal protein loading of samples.

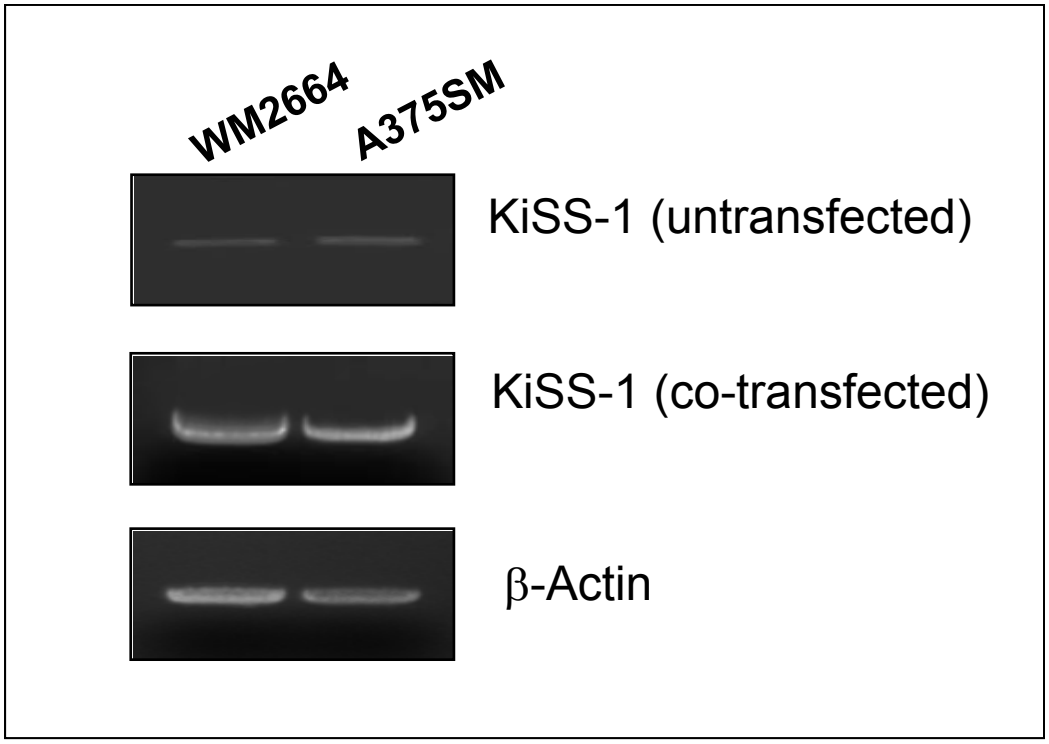

Figure 16: Co-Transfection of DRIP-130 and Sp1 in Highly Metastatic Melanoma Up-regulates KiSS-1 Expression. Co-transfection of WM-2664 and A375SM cells with DRIP-130 and Sp1 resulted in higher KiSS-1 mRNA expression (middle) in RT-PCR analysis as compared with the untransfected melanoma lines (top).

Determination of the KiSS-1 Promoter Region Responsive to Transcriptional Up-regulation by DRIP-130 and Sp1

To demonstrate the importance of the first 100 bases of the KiSS-1 promoter in mediating the effects of Sp1 and DRIP-130, we measured the levels of luciferase induction by constructs driven by just the first 100bp of the KiSS-1 promoter or by a promoter construct with targeted deletion of this first 100bp. Results showed that the 100bp region just proximal to the KiSS-1 transcriptional start site was sufficient for the transcriptional activation of the KiSS-1 promoter mediated by DRIP-130 and Sp1 (Fig. 17). Deletion of the 100bp region of the KiSS-1 promoter resulted in the loss of response to Sp1 and DRIP-130 transcriptional regulation (Fig. 17, last three columns). Additionally, these results suggest that while many putative Sp1 sites are located throughout the full-length human KiSS-1 promoter, the transcriptional effects of DRIP-130 and Sp1 expression may be restricted to just that sequence within the first hundred bases of the promoter.

To further support the significance of Sp1 binding elements localized within the 100bp region proximal to the KiSS-1 start site, EMSA was performed to determine whether these sites were capable of binding the Sp1 transcription factor. Within the first hundred base pairs of the human KiSS-1 promoter sequence, a single GC-rich region spanning +93 to +58 and consisting of approximately ten overlapping putative Sp1 elements stood out as the likely candidate for modulating DRIP-130 and Sp1 up-regulation of KiSS-1 gene as identified by TESS computer analysis. Thus, this region was initially tested for Sp1 binding. WM2664 cells, which were transfected to overexpress DRIP-130 and Sp1, were lysed and incubated with a radiolabeled probe spanning fifty bases of the promoter

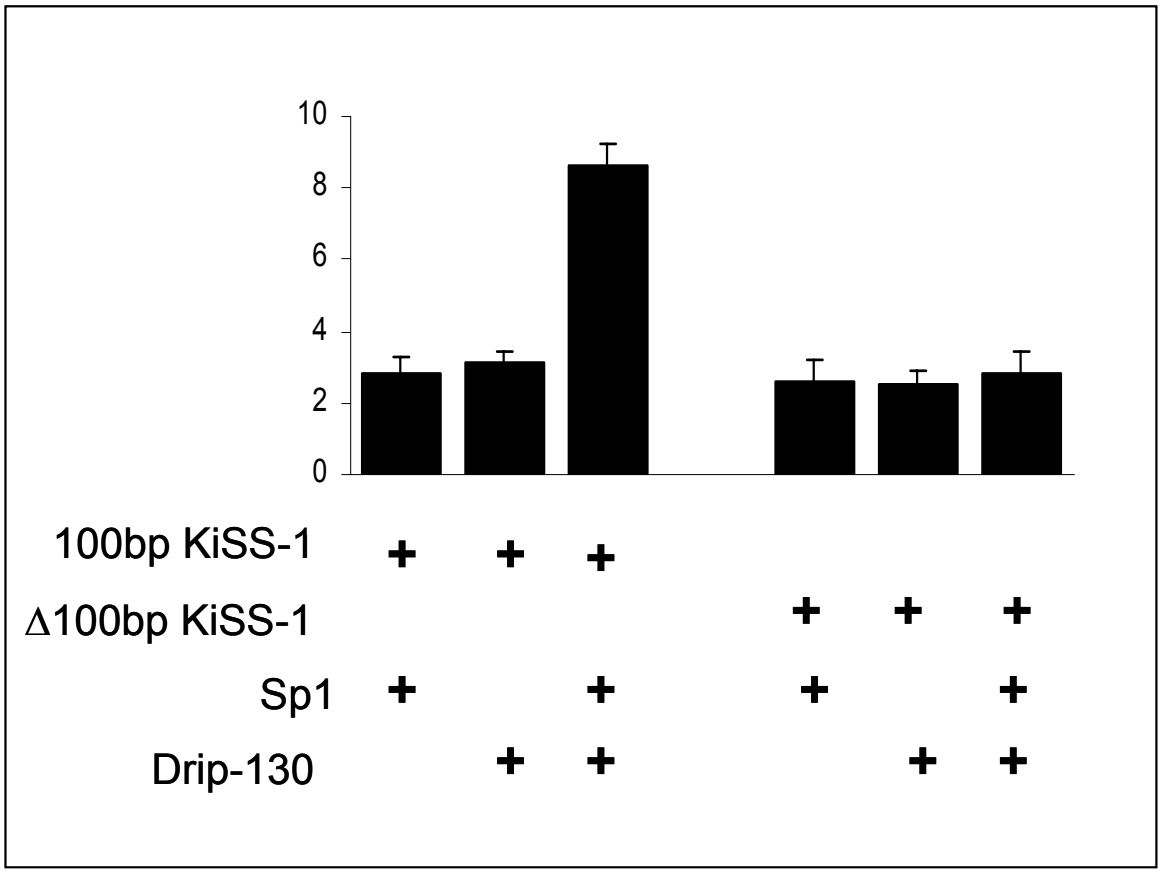

Figure 17: Sp1 Cluster in the First 100bp of KiSS-1 Promoter is Essential for Modulating DRIP-130/Sp1-Mediated Activity. Targeted deletion of 100bp region of KiSS-1 promoter containing Sp1 cluster losses its response to activation by Sp1 and DRIP-130, Expression of a 900bp KiSS-1 promoter-driven luciferase construct lacking the first 100bp (Δ100bp KiSS-1) eliminates DRIP-130/Sp1 modulation, when expressed in malignant melanoma WM2664.

sequence including the candidate GC-rich region. Upon electrophoresis of the proteins and phosphor screen visualization, the patterns of bands revealed that Sp1 formed a DNA-protein complex in the EMSA assay (Fig. 18, lane 2). Incubation of an unlabeled probe competed for protein binding, resulting in a reduction of DNA-protein complex and band intensity (Fig. 18, lane 3). Only the Sp1-specific antibody was capable of super shifting the DNA-protein complex (Fig. 18, lane 5) while the control anti-IgG antibody has no effect (Fig. 18, lane 4), suggesting Sp1 protein interaction with the labeled sequence of the KiSS-1 promoter. The negative control in which labeled probe is seen in the absence of nuclear extract is shown in Figure 18, lane 1. EMSA results using Sp1/DRIP-130 co-transfected A375SM cells showed similar results (data not shown), suggesting that direct interaction of Sp1 occurs within this short 50bp region, specifically the 34 bases identified through computational analysis to consist entirely of overlapping Sp1 sites.

DRIP-130 and Sp1 Complex Co-Precipitate with Chromatin at GC-rich 34-bp Region of KiSS-1 Promoter in Melanoma

As DRIP-130 is known to be a component of the larger CRSP co-activator complex required for Sp1 transcriptional activation, the ability of DRIP-130 to interact with the chromatin bound 100-bp fragment of the KiSS-1 promoter was examined. Chromatin immunoprecipitation (ChIP) analysis in which sheared DNA isolated from DRIP-130/Sp1 co-transfected WM2664 melanoma cells was immunoprecipitated overnight using antibodies specific to DRIP-130, Sp1, and IgG. Precipitates were then washed. Primers, which were designed to overlap the Sp1 rich cluster between +1 and +150, were used to amplify the fragment of the immunoprecipitated chromatin (Fig. 19A). Results showed

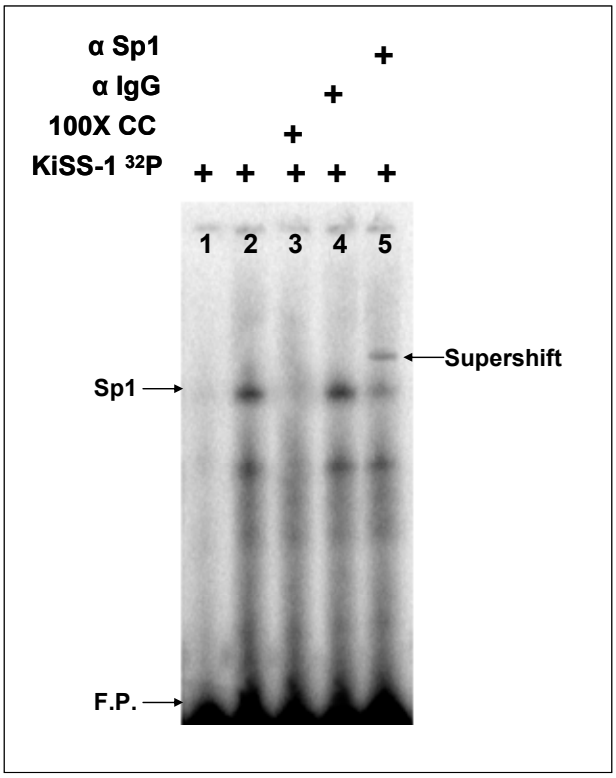

Figure 18: EMSA Reveals Sp1 Interaction with the Sp1 Cluster. This DNA-protein interaction is specific for Sp1 (lane 5), as anti-IgG was incapable of supershifting the bands (lane 4). Additionally, competition for protein binding was shown using unlabeled probe (100X CC; lane 3). Probe controls in the presence (lane 2) and absence (lane 1) are also shown. (C) Primers spanning the first 156bp of the KiSS-1 promoter were designed for chromatin immunoprecipitation analysis (CHiP) to determine if Sp1 and/or DRIP-130 antibodies could precipitate the Sp1 cluster-containing portion of the chromatin-bound KiSS-1 promoter.

Figure 19: ChIP Analysis Reveals Specific Interactions of Sp1 and DRIP-130 with the KiSS-1 Promoter. ChIP demonstrated specific binding of Sp1 and DRIP-130 proteins (lanes 4 and 5, respectively) to the chromatin-bound KiSS-1 promoter in WM2664 cells. Precipitation using an IgG-specific antibody was used as a negative control (lane 3), whereas, the positive control for the PCR reaction using a KiSS-1 promoter expressing construct is seen in lane 1. A portion of the chromatin-bound promoter prior to antibody incubation was also used as a control for the PCR reaction (lane 2).

that the antibody to Sp1 was able to immunoprecipitate the first 150-bp of the chromatin-bound KiSS-1 promoter (Fig. 19B, *lane 4*). However, the antibody to IgG, used as a negative control, did not precipitate this region of the KiSS-1 promoter and thus did not form PCR product (Fig. 19B, *lane 3*). Controls included the PCR products using both the non-immunoprecipitated input *(lane 2)* and a vector containing the full-length KiSS-1 promoter sequence *(lane 1).* Furthermore, the antibody to DRIP-130 immunoprecipitated this region of the KiSS-1 promoter (Fig. 19B, *lane 5*). Previous incubation of DRIP-130 specific antibody in EMSA assays showed no conclusive DNA-protein interaction (data not shown), however, such seemingly inconsistent findings may likely result from even very small differences in the antibody or protein binding conditions in EMSA and CHiP assays. Generally, however, CHiP assays are considered the most sensitive and CHiP assays using A375SM cells were consistent with the results from WM2664 cell line. From our CHiP assays, we conclude that DRIP-130 exist in a protein complex that binds to the Sp1 cluster between +93 and +58 in the chromatin-bound form.

Co-Transfection of Metastatic Melanoma Cell Lines with DRIP-130 and Sp1 Results in Reduced Migratory and Invasive Properties

KiSS-1 is known to inhibit migration and invasion when overexpressed in NIH3T3 cells and other cells (Stafford et al., 2002; Lee and Welch, 1997a). Considering that co-transfection of both DRIP-130 and Sp1 resulted in an increase of KiSS-1 expression as evaluated by RT-PCR (Fig. 15), functional assays measuring the degree of invasive and migratory behavior of melanoma cells were used to determine whether re-introduction of DRIP-130 and Sp1 in highly metastatic melanoma was sufficient to induce the metastatic

inhibition seen with KiSS-1 overexpression. Wound-healing assays using WM2664 cells transfected with vector-only (Fig. 20A), Sp1 (Fig. 20C), DRIP-130 (Fig. 20D), and Sp1 and DRIP-130 (Fig. 20E), were used to determine migratory ability of cells upon treatment. Scratches across each well were made with a pipette tip 36 hours following the initial transfection when cells grew confluent. After 24 hours incubation, cell migration within each well was compared to a KiSS-1 transfected positive control (Fig. 20B). A typical freshly made wound upon washing once with PBS before incubation was shown in Fig. 20F). Results show that only DRIP-130/Sp1-cotransfected (Fig. 20E) and KiSS-1-transfected (Fig. 4B) melanoma cells show any significant inhibition to cell migration after 24 hrs upon injury. DRIP-130-transfected (Fig. 20D) and vector-transfected cells (Fig. 20A) showed a good similar amount of wound healing, whereas, Sp1-transfected cells seemed to show slightly greater migratory capacity (Fig. 20C). In addition, Boyden Chamber invasion assays using similarly transfected cells revealed that cell over-expressing DRIP-130 and Sp1 modulated anti-invasive behavior comparable to KiSS-1 transfected cells (Fig. 21G). These functional assays suggest that re-introducing DRIP-130 and Sp1 into highly metastatic melanoma cells will result in reduced invasive and migratory behavior similar to the anti-metastatic property produced upon KiSS-1 over-expression in the melanoma cells.

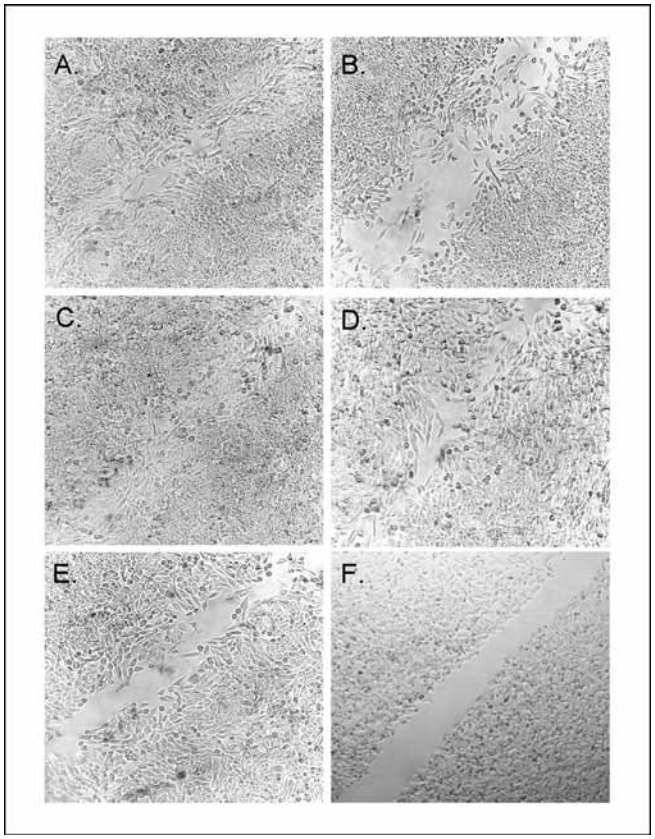

Figure 20: Co-Transfection of DRIP-130 and Sp1 Induces Anti-Migratory Characteristics in Metastatic Melanoma. (A-F) Wound healing assays were used to determine if over-expression of Sp1 and DRIP-130 in melanoma cells would mimic the anti-migratory properties seen in KiSS-1 transfected cells. WM2664 cells were singly transfected with Sp1 or DRIP-130 (C and D, respectively) and compared to the vector-transfected negative control with little or no significant difference (A). Likewise, melanoma was co-transfected with Sp1 and DRIP-130 (E) and showed significant inhibition of cell migration as compared to the negative control (A). Wound healing assays were also done on KiSS-1 expressing cells as a positive control (B). A typical wound seen after washing with PBS before overnight incubation (F).

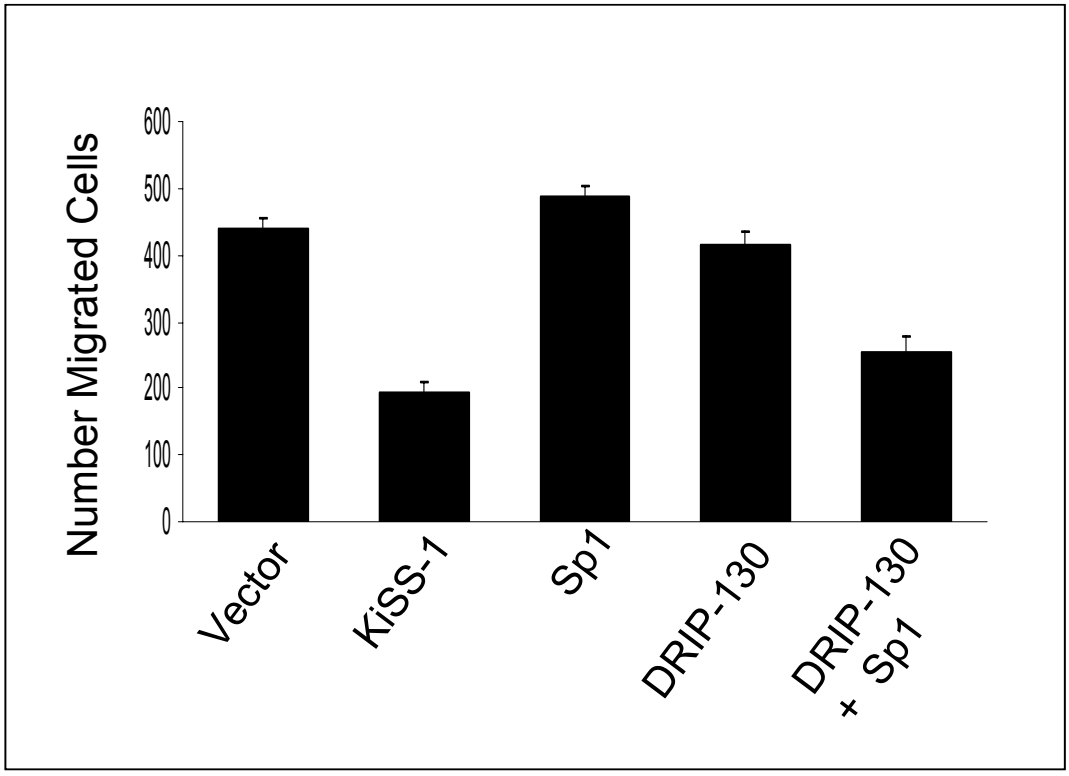

Figure 21: Co-Transfection of DRIP-130 and Sp1 Induces Anti-Invasive Characteristics in Metastatic Melanoma. Results from Boyden chamber migration assays demonstrate that co-transfection of Sp1 and DRIP-130 leads to significant migratory inhibition compared to single transfections, similar to the inhibitory effect of KiSS-1 overexpression in the cells.

Discussion

Loss of KiSS-1 expression has been repeatedly found in the progression of multiple forms of cancer metastasis and thus, the degree to which it is lost has become a fairly accurate indicator of the severity of tumor metastasis (Lee and Welch, 1997b; Sanchez-Carbayo et al., 2003; Masui et al., 2004; Ikeguchi et al., 2004.; Kotani et al., 2001). Re-introduced expression of KiSS-1 in highly metastatic cells which have progressively lost KiSS-1, has been shown to inhibit migration and invasion of cells and cancer metastasis (Lee and Welch, 1997b; Muir et al., 2001; Ohtaki et al., 2001; Kotani et al., 2001; Stafford et al., 2002). Considering the importance of KiSS-1 expression in gauging metastasis, an understanding of the transcriptional regulation of KiSS-1 and the mechanism whereby it is lost during the progression of cancer is essential. Here we demonstrate that KiSS-1 transactivation is controlled not only by the transcription factor, AP-2α, as previously published in breast cancer cells (Mitchell et al., 2006), but also by the Sp1 co-activator complex DRIP-130. Specifically, loss of DRIP-130, one of 15 subunits which collectively forms the CRSP co-activator (Taatjes and Tjian, 2004), leads to the loss of KiSS-1 transcriptional activation in metastatic melanoma cells.

DRIP-130 was originally identified along with the other subunits of CRSP as being essential for Sp1-mediate gene transcription (Ryu et al., 1999). In addition, other labs have found a correlation between loss of DRIP-130 and loss of KiSS-1 expression, although the exact mechanism behind this correlation was never made (Goldberg et al., 2003). The genomic region encoding DRIP-130, the short arm of human chromosome 6 (6q16.3-q23), is commonly lost as a result of the progression of melanoma. In addition, this same region

of chromosome 6 encodes AP-2α, the expression of which has previously been shown to regulate transcription of KiSS-1 in breast cancer, suggesting that loss of this region of the genome is a key milestone in the loss of KiSS-1 expression and heightened metastasis (Goldberg et al., 2003; Mitchell et al., 2006). Although the GC-rich *KiSS-1* promoter contains multiple Sp1 sites, serial truncations of the *KiSS-1* regulatory region revealed that the site responsible for modulating DRIP-130/Sp1 transcriptional regulation was localized to the first 100-bp (Fig. 13). This region contained multiple overlapping Sp1 sites, and its deletion led to a total lack of transactivation upon co-transfection with DRIP-130 and Sp1 (Fig. 17). Later, the ability of both a Sp1-specific antibody to supershift the discrete 34-bp region just proximal the KiSS-1 transcription start site in EMSA assays indicated that this element may modulate Sp1 transactivation through action of DRIP-130. Chromatin immunoprecipitation (CHiP) using this region of the promoter also demonstrated that Sp1 and DRIP-130 were capable of forming a complex with the promoter region (Fig. 19A and 19B). Although targeted mutations of the overlapping Sp1 sites were attempted, due to the high GC-content, we found that generation of 100-bp constructs bearing such mutations to the KiSS-1 promoter was not feasible, therefore we are unable to determine which Sp1 elements, or whether all elements in the 34bp Sp1 cluster are essential for Sp1-mediated transcriptional control.

The data presented here, as well as data from previous publications, indicates the overall importance of the genetic material on the small arm of chromosome 6 (6q16.3-q23) in maintaining KiSS-1 expression (Goldberg et al., 2003; Mitchell et al., 2006). Additionally, it suggests a possible mechanism for loss of KiSS-1 during tumor metastatic

progression, by which gross chromosomal loss of the genetic material on chromosome 6q16.3-q23 encoding both AP-2α and DRIP-130 in melanoma, leads to reduced KiSS-1 expression (Fig. 22). In normal cells, DRIP-130 is expressed properly and interacts with other proteins in the CRSP complex that helps form the larger Mediator complex which binds TFIID to regulate Sp1-mediated transcriptional regulation (Fig. 22A). Mutation or loss of the genomic portion of chromosome 6q16.3-q23 encoding DRIP-130 results in loss of KiSS-1 expression through failure of proper CRSP and Mediator complex formation (Fig. 22B), resulting in loss of metastatic inhibition seen in metastatic melanoma lacking KiSS-1 expression.

The transcription factor Sp1 regulates multiple genes expressed in both normal and tumorigenic tissues (Lania et al., 1997; Suske, 1999; Black et al., 2001; Safe and Abdelrahim, 2005). Genes involved in cell cycle progression, cell growth and differentiation, apoptosis, and capillary growth have all been shown to contain GC-rich Sp1 elements that regulate their expression (Ryuto et al., 1996; Finkenzeller et al., 1997; Ji et al., 1997; Dong et al., 1999). Recent studies suggest that Sp1 expression may also be a determining factor in tumor metastatic progression; such studies indicate that higher Sp1 levels correlate directly to severity of gastric, colonic, breast, thyroid and pancreatic cancers through their transactivation of vascular endothelial growth factor, insulin-like growth factor I, sodium-iodide symporter, and E-cadherin (Wang et al, 2003; Yao et al., 2004; Jiang et al., 2004; Wang et al., 2005; Shi et al., 2001; Abdelrahim et al., 2005; Zhu et al., 2002; Liu et al., 2005). Another explanation for increased Sp1 in such cases, however, is that the normal ratio of Sp1 expression is altered such as in the overexpression of

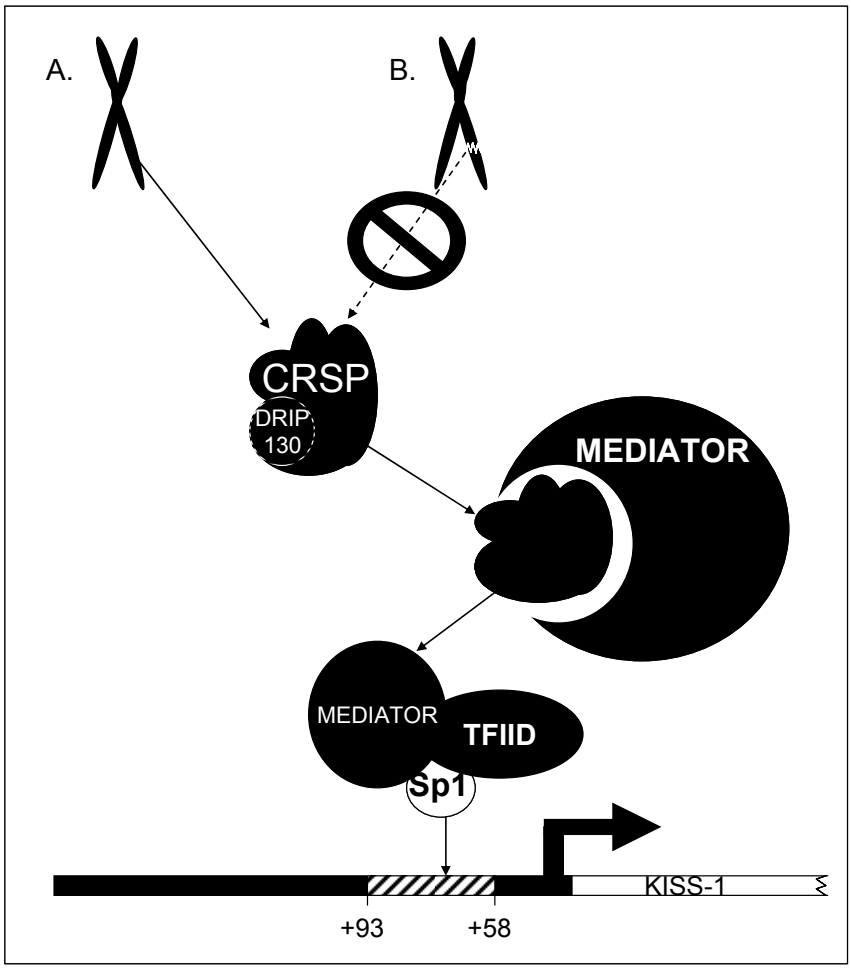

Figure 22: Schematic model of DRIP-130/Sp1-Mediated KiSS-1 transcriptional regulation. In normal skin cells in which chromosome 6 is intact (A), DRIP-130 is expressed properly and interact with other protein subunits in the CRSP complex that helps form the larger Mediator complex which binds TFIID to regulate Sp1-mediated transcriptional regulation of KiSS-1 gene. (B) Mutation or loss of the genomic portion of chromosome 6q16.3-q23 encoding DRIP-130 results in loss of KiSS-1 expression through failure of proper CRSP and Mediator complex formation, resulting in loss of metastatic inhibition seen in metastatic melanoma lacking KiSS-1 expression.

thrombin receptor (PAR-1) in highly metastatic melanoma (Tellez et al., 2003). *Tellez et al.*, explains how an irregular ratio of AP-2α/Sp1 in which AP-2 is lost leads to the heightened expression of PAR-1. Considering that Sp1 levels remain relatively constant in both normal and metastatic melanoma (Fig. 15), a similar mechanism by which the DRIP-130/Sp1 ratio is reduced may likewise account for the loss of key genes in tumor metastasis suppression and increased tumor metastasis. Such an increase in metastasis may occur not only through loss of DRIP-130 co-activation of KiSS-1, but also through increased Sp1 modulated activation of pro-metastatic genes.

Although our lab previously found that AP-2α is a key regulator of KiSS-1 in metastatic breast cancer cells, our data now indicates the presence of a second gene, DRIP-130, that localizes to the same chromosomal region as AP-2α, and similarly regulates the transactivation of the KiSS-1 promoter (Mitchell et al., 2006). Additionally, our data supported previous studies suggesting a correlation between loss of KiSS-1 expression and loss of DRIP-130 in metastatic melanoma cells and we have localized the responsive region of the promoter to within 34-bp of KiSS-1 promoter. Considering the growing clinical importance of KiSS-1 as an effective suppressor of metastasis, our studies suggest a mechanism for its regulation in normal tissues and offer a likely mechanism for its loss during progression of cancer metastasis.

CHAPTER V

INTRODUCTION TO THE FUNCTION OF SMALL GTPASES IN THE LENS

Introduction to Small Molecule Rho GTPases

Small molecule GTPases comprise nearly 50 different families of proteins, all of which function as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state capable of signaling downstream effectors. The ratio of GTP to GDP within the cell determines the activity of these small molecule GTPases, as do the regulators which govern their action. Three major types of GTPase-binding proteins are known, guanine nucleotide exchange factors which promote the exchange of GDP for GTP, GTPase activating proteins which enhance the intrinsic GTPase activity of the proteins leading to their inactivation, and guanine nucleotide dissociation inhibitors which inhibit GTPase activity and also prevent the exchange of GDP for GTP. Of the many known small molecule GTPases, the Rho family of proteins is one of the most heavily studied. The Rho family consists of Rho (RhoA, RhoB, and RhoC), as well as Rac (Rac1, Rac2 and Rac3), Cdc42, TC10, TCL, Wrch1, Chp/Wrch2, RhoG, RhoH/TTF, and Rnd (Rnd1, Rnd2, and Rnd3/RhoE) of which the effects of Rho, Rac and Cdc42 have been characterized most extensively.

The functions of RhoA, Rac1 and Cdc42 were originally determined when their expression was found to influence the formation of F-actin structures in Swiss3t3 fibroblasts. Upon LPA induction, the fibroblasts formed actin stress fibers and focal adhesions, which could be prevented by the addition of Clostridium botulinum C3

exoenzyme, an enzyme which ADP-ribosylates asparagine-41 inactivating Rho GTPase and causing depolymerization of the actin cytoskeleton. Since then, multiple stimuli have been found to induce Rho family activation. Growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and insulin induce Rac1-mediated membrane ruffling, cell-cell adhesions and actin polymerization (Kaibuchi et al., 1999; Ridley and Hall, 1992), whereas bradykinin induces microspike or filopodia formation via Cdc42 activation (Ridley and Hall, 1992). In addition to the cytoskeletal rearrangements modulated by the Rho family, these proteins have also been found to regulate multiple biochemical processes upon stimulation of upstream membrane receptors, including G1-cell cycle progression, membrane trafficking, nuclear factor NF-κB (Perona et al., 1997) and serum response factor transcription factors (Hill et al., 1995), NADPH oxidase complex (Abo et al., 1991), the c-jun N-terminal kinase (JNK) (Minden et al., 1995) and p38 mitogen activated protein kinase pathways (Coso et al., 1995). The deep involvement of the Rho family of GTPases in nearly every major pathway underscores the importance of these proteins as key signaling intermediaries.

Rho Family in the Lens

Small GTPases were originally implicated in the processes involved in lens cell development and proliferation nearly a decade ago when their presence was found to be highly enriched in insoluble lens fractions using GTP overlay assay (Rao et al., 1997b). A combination of two-dimensional electrophoresis and GTP overlay identified over 25 small molecule GTPases between 20-30 kDa, including both Rho and Rac. Additionally, incubation of lens fractions with C3-exoenzyme confirmed the presence of Rho in lens

lysates by their ability to become ADP-ribosylated in vitro (Rao et al., 1997b). The abundance of Rho family proteins in the lens suggested they may play important roles in actin rearrangement, integrin signaling, cellular differentiation, or other cellular processes in which Rho GTPases are known to be intricately involved. No direct influence of the Rho family on lenticular differentiation, function and structure, however, was seen until just a decade ago when studies showed that treatment of lenses with lovastatin, a cholesterol-reducing agent, was also was found to prevent iso-prenylation of Rho proteins, effectively blocking Rho recruitment to the membrane (Rao et al., 1997a). Lovastatin was found to induce aberrant cell proliferation and decreased lens transparency leading to cataract formation via its ability to inhibit Rho signaling. Lovastatin treatment of both human and rabbit lenses resulted in overall deterioration of the lenticular epithelium, increased vacuole development in the cortex, and distortion of elongating lens fiber cells (Rao et al., 1997a). Since then, further studies have illustrated the importance of expression of Rho proteins during eye development and maintenance. As Rho GTPases have been found to be expressed in lens tissue, it is likely that their activity may influence lens cell differentiation and cytoskeletal morphology, and possibly lens transparency as well.

Although once thought to be ubiquitously and uniformly expressed, recent evidence suggests that the Rho family of GTPases are commonly differentially expressed and that such unique patterns of expression, both spatial and temporal, are necessary for the physiological activities of the Rho family proteins. Such differential distribution has already been found in the rat hippocampus, cerebellum and neocortex (O'kane et al., 2003;

Olenik et al., 1999) and implies that these proteins have unique effects and are required during specific periods of development. The differential expression patterns of the Rho proteins in the eye may indicate that they are responsible for different aspects of lens cell function and maintenance. Differential expression of the Rho family of GTPases has been found in the chick retina in which these proteins were expressed differently during different developmental stages (Santos-Bredariol et al., 2002). Additionally, RhoB was found to be almost exclusively in the lens after 11.5E day and was only strongly expressed between postnatal days 7-18 in the retina suggesting it is required only during lens fiber elongation and in the development of the retina (Maddala et al., 2001a).

Upstream Activators of Rho Family GTPases: Growth Factor Mediated Cell Signaling through the Rho family

The Rho family of GTPases primarily work to transduce the effects of extracellular signals to downstream modulators of the actin cytoskeleton through membrane receptors. Growth factor receptors commonly mediate signals to Rho, Rac and Cdc42 by activating guanine exchange factors, or alternatively, by inhibiting guanine activator proteins, therefore activating the GTPase signaling pathway. Rho was first found to be antagonized upon treatment with lysophosphatidic acid (LPA), which induced the formation of the actin stress fibers in Swiss3T3 cells. This response results from LPA signaling via multiple G-protein coupled receptors (Ridley and Hall, 1992; Goetzl and An, 1998). Lysophospholipid, is a bioactive lipid growth factor which regulates multiple processes involved in cytoskeletal reorganization, including proliferation, adhesion and migration. Previous immunohistochemistry in the lens revealed that both LPA and sphinogosine-1

phosphate receptors were expressed strongly in cortical actin stress fibers as well as in focal contacts of serum-starved lens epithelial cells as active Rho GTPase levels were increased (Okamoto et al., 2000). This effect of LPA on cytoskeletal organization in the lens appeared to be similar to the effects of b-FGF, transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF), suggesting a potential role for this lipid agonist in lens epithelial cell migration, proliferation, and cell survival (Maddala et al., 2003). Both LPA and thrombin are known to signal through Rho GTPase mediated pathways in order to effect cytoskeletal reorganization (Hall, 1998; Van Aelst and D'Souza-Schorey, 1997; Essler et al., 1998; Jalink et al., 1994). Active thrombin receptor has also been identified and characterized in the human lens (James et al., 2005) and considering that thrombin induces rapid geranylgeranylation of RhoA, leading to its activation in endothelial cells, it may prove equally as important in lens tissues (Ohkawara et al., 2005).

Fibroblast growth factor (FGF) is solely responsible for differentiation of lens epithelial cells into lens fiber cells (de Iongh et al., 1997) and is constantly expressed throughout normal eye development and maintenance. Although TGF-β and FGF have been shown to have opposing effects on lens cell proliferation in vitro, as TGF decreases whereas FGF increase cell number (Nishi et al., 1996; Ueda et al., 2000), FGF has also been found to aggravate the effect of TGF-β possibly by inducing cell proliferation when concomitantly repressing TGF-β-stimulated cell death (Cerra et al., 2003) . Additionally, FGF-2 expression is required for increased cell proliferation in injured lens cells in vitro, suggesting it has an additional role in cytoskeletal rearrangement during wound healing (Tanaka et al., 2004).

Transforming Growth Factor-β Signaling via Rho GTPases

TGF-β has been implicated in multiple human diseases (Blobe et al., 2000), including lenticular anomalies resembling those characteristic of posterior capsule opacification and subcapsular cataract development (Liu et al., 1994; Hales et al., 1995; Srinivasan et al., 1998; Hales et al., 1999; de Iongh et al., 2001). Overexpression of TGF-β in rat model systems has shown that TGF-β is responsible for the epithelial-mesenchymal transition that is characteristic of cataratogenesis and precedes expression of collagen type I, alpha-smooth muscle actin, fibronectin, and tanascin, hallmarks of cataractous lens tissue (Cerra et al., 2003; Marcantonio and Reddan, 2004). TGF is strongly expressed in lens epithelium, and is expressed in a latent form in the ocular media (Jampel et al., 1990; Ochiai and Ochiai, 2002). Although the exact mechanism of TGF-β induced Rho activation is unknown, the cytoskeletal rearrangements modulated upon TGF-β treatment are known to modify Rho family GTPase activation (Masszi et al., 2003). Rho may mediates TGF-β induced epithelial-mesenchymal transition in part by stimulating the cytoskeletal rearrangement through activation of myosin light chain or inhibition of myosin light chain phosphatase via the Rho kinase, in addition to activation of serum response factor-mediated transactivation of the alpha smooth muscle promoter (Masszi et al., 2003).

Cataract removal commonly induces a wound healing response marked by opacification of the remaining lens capsule (Tanaka et al., 2004). This response often stimulates expression of extracellular matrix proteins and integrins, as well as structural elements. TGF-β modulates multiple biological processes including apoptosis, cell

proliferation, migration and adhesion (Lee and Bae, 2002; Zhu et at., 2005). TGF-β is also believed to play a principle role in initiating cell signaling in response to lens injury and is highly expressed in the aqueous humor of the human eye (Marcantonio and Reddan, 2004). Thus, signaling mediated through the transforming growth factor receptor has roles both in the normal processes involved with lens fiber differentiation, as well as inappropriately during epithelial-mesenchymal transition and is similar to certain types of cataracts including anterior subcapsular and posterior capsular opacification, which develop after cataract surgery (Gordon-Thomson et al, 1998; Wormstone et al., 2002). Posterior capsule opacification is known to result when cell proliferation, matrix deposition, and capsular wrinkling cause increased scatter of light through the lens (Marcantonio and Reddan, 2004). TGF-β2 has been found to influence alpha5-β1 integrin distribution in vitro (Liu et al., 1994; Lee and Joo, 1999; Wormstone, 2002). And, in vivo experiments in which exogenous TGF-β is added show a marked increase in the incidence of cataractous changes (Hales et al., 1999). TGF-induced cataract formation also results from stimulation of the Smad signaling pathways (Saika et al., 2001; Wormstone et al., 2004). Smads transduce signals from TGF-β receptors on the cell surface to the nucleus where they bind as a complex to certain promoter regions where they regulate transcriptional activity (Piek et al., 1999; Massague and Wotton, 2000). Activation of the TGF-Smad pathway leads to increased apoptosis and decreased cell proliferation (Chen et al., 2001). Using breast epithelial cells as a model system, Rho signaling has been shown to be essential in mediating TGF-β induced growth inhibition (Kamaraju and Roberts, 2005). Activation of Rho kinase leads to phosphorylation of serine residues in the linker regions of Smad2 and

Smad3, leading to Smad activation, resulting in down-regulation of c-myc and up-regulation of p21waf1 causing growth suppression and loss of cell proliferation (Kamaraju and Roberts, 2005). Contrarily, Cdc42 is activated by Smad7, a negative regulator of the TGF-β-Smad signaling pathway in breast carcinoma and prostate epithelial cells which leads to actin cytoskeletal rearrangement, inhibition of Smad2 and Smad3 phosphorylation and ubiquitination of the cognate TGF-β receptor (Edlund et al., 2003; Kavsak et al., 2000; Ebisawa et al., 2001). As RhoA commonly antagonizes the effects mediated by Rac1 and Cdc42, it is possible that whereas RhoA mediates the initial wound response in the lens via Smad transcriptional modulation, Smad induced Cdc42 expression may be concomitant with later actin filament reorganization through activation of p38 pathway (Edlund et al., 2004). Although TGF-β signaling is principally through the activation of Smad proteins, Smad-independent TGF-β signaling pathways have also been identified. TGF-β treatment of rat basophilic leukemia and human prostate epithelial cells results in a rapid (within 5-10 minutes) induction of lamellipodia formation through activation of Rho GTPases (Edlund et al., 2002). This is then followed by conventional TGF-β signaling through phosphorylated Smads leading to formation of actin stress fibers.

Role of Rho Family GTPases in Lens Cell Adhesion

The lens is composed of a single epithelial layer. Lens epithelial cells located at the equatorial region migrate posteriorly, bringing them into contact with the vitreous humor which contains factors which induce cells to terminally differentiate into fiber cells. This process is continuous. Over the course of a lifetime, the newly differentiated fiber cells displace older fiber cells pushing them towards the center of the lens. The oldest of these

cells, the nucleus, thus represents those cell which composed the earliest embryonic lens, and the peripheral region, the cortex, consists of the biologically active portion of the lens. The lens thus consists primarily of two distinct cell types: lens epithelial cells which are situated at the anterior region of the eye, and the lens fiber cells, which are found in the cortical and posterior regions. Normal differentiation of the lens epithelial cells involves loss of cuboidal shape characteristic of epithelial cells and the adoption of a more elongate, ribbon-like morphology, as well as the loss of cellular organelles, including the nucleus. The process of elongation, coupled with the directed migration of lens cells requires cells to continuously make and dissolve cell-cell and cell-matrix adhesions and generate contractile forces. Aberrations in these processes may lead to irregular lens shape and function. As the Rho family is intricately involved in these processes, they are likely to be key regulators of structural signaling in the lens.

The first step in the process of epithelial cell migration involves extension of the lamellipodia or filopodium at the leading edge primarily by the action of Rac1 and Cdc42. Further, extension is regulated by G-proteins, as well as actin-capping proteins (Etienne-Manneville and Hall, 2002). Following the initial elongation, integrins bind protein in the extracellular matrix (ECM) to form weak attachments, which are quickly reinforced by the formation of focal adhesions which incorporate both signaling and scaffolding proteins. Nuclei and cytoplasmic migration is mediated by myosin dependent contraction of the actin cytoskeleton. Disassembly of focal contacts at the trailing edge of the cell then releases the cell from the ECM and allows the trailing end to be retracted into the body of the cell. Adhesion and migration requires a high degree of coordination since these two

processes may be seen to act contrary to one another, in that a high level of adhesion will inhibit migration by preventing detachments, and low adhesion may also hamper migration by not providing sufficient traction for movement. Epithelial cell migration differs from migration of fibroblasts in that rather than migrating individually, cells migrate as sheets, thus maintaining the barrier quality of epithelium. Additionally, epithelial cells do not form focal adhesions although they express those proteins which constitute focal contacts and are used in creating cell-ECM connections. The contractile force that propels the epithelial sheet is provided by actin fibrils found perpendicular to the migrating cells (Fenteany et al., 2000). As the connection with the ECM provides the migratory force, abnormal in the composition of the ECM can interfere with such interactions and lead to defects in migration. In the lens, the lens capsule provides the ECM and is composed of fibronectin, collagen IV, and laminin (Cammarata et al., 1986).

Integrins modulate signals from the ECM to the cell's interior which may affect survival, proliferation, and cell differentiation. Signals may also be mediated outwardly from within the cell and thus may affect migration of the cell by modulation of the extracellular matrix. Modulation of the actin cytoskeleton is primarily resultant of Rho GTPase signaling, as its downstream effects Rho kinase and LIM kinase alter actin-based cytoskeletal structures through modulation of myosin light chain and myosin light chain phosphatase activation. While the initial clustering of integrins into focal adhesions is Rho independent, cell contraction through activation of Rho proteins leads to further aggregation of integrins and the formation of larger focal adhesions (Machesky and Hall, 1997; Chrzanowska-Wodnicka and Burridge, 1996; Clark et al., 1998). In the lens,

supplementation of cultured human lens epithelial cells with growth factors including EGF, b-FGF, PDGF, LPA and TGF-β results leads nearly immediate increases in Rho GTPase activation which were maintained for nearly an hour post-treatment (Maddala et al., 2003). Whereas b-FGF, PDGF, and TGF-β induced marked increase in actin-based cytoskeletal reorganization, addition of EGF, which like the other growth factors was found to activate both Rho and Rac, elicited weak stress fiber formation but significant membrane ruffling and focal adhesion formation (Maddala et al., 2003). This response to growth factors present in both aqueous and vitreous humors may influence lens epithelial differentiation as well as proliferation and migration by mediating the formation of cell-ECM interactions critical for lens cell survival (Menko et al., 1998; McAvoy et al., 1999; Maddala et al., 2003). Treatment of cell with C3-exoenzyme and a Rho kinase inhibitor, Y-27632, inhibited focal adhesion formation, thus supporting the essential role of Rho and its downstream effectors in mediating this response (Maddalla et al., 2003). Additionally, recent characterization of a transgenic mouse model in which C3-exoenzyme was specifically expressed in the lens under the α-crystallin promoter, which functionally knocks out Rho GTPase activity in the lens, reveals that lenses from these mice demonstrate aberrant membrane architecture and altered gene expression patterns in which structural proteins and cell survival proteins are deregulated along with higher incidence of cataract formation, confirming the critical role of GTPases play in normal lens proliferation, differentiation, and function (Maddala and Rao, 2005).

However, during the formation of ASC and PCO types of cataracts, lens epithelial cells differentiate into myoblast or mesenchyme-like cells (Wride and Sanders, 1998; Lee

and Joo, 1999) which require expression of cell adhesion molecules, including integrins to mediate both cell-cell and cell-ECM interactions. Multiple integrins are shown to be highly expressed in human cataracts including β1, β2, α2 and α5, whereas others such as a6, a key regulator of lens cell differentiation may be downregulated (Zhang et al., 2000; Lim et al., 2001). Processes involving adhesive interactions are also capable of modulating actin-based cytoskeletal rearrangement which involves activation of the Rho family of GTPases (Clark et al., 1998). Integrins are membrane receptors located in discrete regions of the cell's surface termed 'focal adhesions' that link the extra cellular membrane to collections of intracellular cytoskeletal elements leading to the formation of actin stress fibers (Chrzanowska-Wodnicka and Burridge, 1996; Craig and Johnson, 1996; Jockusch and Rudiger, 1996). Additionally, integrin-mediated cellular adhesion can induce formation of other actin structures including membrane ruffles, and microspikes and other physiological modifications leading to cell spreading and motility (Craig and Johnson, 1996; Zigmond, 1996).

Downstream Effectors of Rho Family GTPases in the Lens

Activation of the Rho family of GTPases has also been found to influence transcription activation as well as cell cycle progression through activation of its downstream effectors, serum response factor (SRF), the CDK kinases, and cell cycle inhibitor expression. RhoA is the most heavily researched member of the Rho family of GTPases, and as such, its involvement in lens development and cellular differentiation is the most highly characterized. Two major downstream effectors are known to modulate the effect of Rho activation on the cytoskeleton; these are the Rho kinases and the

Diaphanous forming subfamily. Rho kinases are serine/threonine kinases that induce reorganization of actin filament bundles by activating myosin light chain (MLC) or inactivating MLC phosphatase through direct phosphorylation of these substrates. (Govek et al., 2005). Additionally, activation of Rho kinase promotes inactivation of the actin depolymerization factor cofilin through LIMK activation leading to accumulation of F-actin. (Sumi et al., 1999; Amano et al., 2001). In addition to its affects on cofilin, MLC and MLC phosphatase, the expression of ROCK has also been found to be inversely correlated to αB-crystallin expression in lens epithelial cells (Khurana et al., 2002).

Additionally, Rho family activation has also been shown to regulate transcriptional modulation as well as cell cycle progression, independently of its influence on the actin-based cytoskeleton. Such effects as these are carried out by the downstream effectors, serum response factor (SRF) and by the cyclin dependent kinases (Cdk) (Maddala et al., 2004). Although little is known about SRF-mediated signaling in the lens, Cdk5 is known to regulate cell-matrix as well as cell-cell adhesions in the lens epithelium (Negash et al. 2002) Cdk5 positively regulates cell adhesion and is usually found at higher expression in the tips of elongating lens fiber cells (Gao et al., 1997; Negash et al., 2002). Cdk5 substrates include c-Src, myosin heavy chain, focal adhesion kinase and p21-activated kinase, all of which are known to regulate different aspects of cell adhesion and migration (Kato and Maeda, 1999; Pato et al., 1996; Xie et al., 2003; Nikolic et al., 1998; Banerjee et al., 2002; Zelenka, 2004). Cdk5, which is controlled by the activation of Rac1, enhances adhesion of both fibronectin and collagen IV and its ability to limit active Src recruitment to the leading edge of corneal epithelium suggests a possible mechanism whereby it

enhances migration by decreasing focal contact stability (Negash et al., 2002; Gao et al., 2004; Frame and Brunton, 2002; Zelenka, 2004). Rac1 and Cdc42 directly activate the mitogen-activated protein kinase pathway through activation of P21-activated kinase. Rho also indirectly activates the Ras signaling pathway, which in turn activates the mitogen-activated protein kinase cascade. Expression studies of the members of the mitogen-activated protein kinase pathways in the lens revealed that extracellular signal-related kinase1 and 2 (ERK 1 and ERK2) were most strongly expressed, whereas jun-N-terminal kinase1 and 2 (JNK1 and JNK2) and p38 kinase were found more highly expressed in the lens epithelium (Li et al., 2003).

Mutations in Rho Modulated signaling Lead to Lenticular Anomalies

Fiber cells highly express crystallin proteins, which are partly responsible for the transparency and reduction of light scatter. However, fully differentiated fiber cells are incapable of replacing damaged regions and have limited ability to fully repair them, thus fiber cells must remain dependent of the surrounding lens epithelium to protect and sustain transparency (Rao et al., 1994). Multiple bacterial toxins are capable of modifying the activity of Rho GTPases. Clostridium difficile toxin B inactivates nearly every member of the Rho GTPase family (Aktories et al., 2000) whereas another toxin isolated from Clostridium botulinum (Aktories, 1997) exhibits more specific activity, inactivating only RhoA, RhoB, and RhoC. As mentioned previously, the functional knockout of Rho in which the C3 exoenzyme was integrated into the control of the crystallin promoter for eye specific expression, showed numerous phenotypic and histological aberrations, indicating the important role of the Rho family in modulate the organization of the cytoskeleton of

the lens (Madalla et al., 2005). Similarly lovastatin blocks Rho signaling is through the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which causes loss of mevalonate, a precursor for the production of farnesyl and geranylgeranyl lipid groups, the binding of which is required for targeting Rho proteins to the membrane (Goldstein and Brown, 1990). Mutations in or loss of mevalonate kinase has also been shown to induce cataractogenesis supporting the claim that proper Rho regulation is essential for lens maintenance (Hoffmann et al., 1986). Additionally, lovastatin treatment along with inducing lens opacification also decrease lens epithelial proliferation indicating a loss of Rho may stimulate apoptotic pathways (Rao et al., 1997b; Coleman and Olson, 2002).

CHAPTER VI

DIFFERENTIAL EXPRESSION OF SMALL MOLECULE RHO GTPASES DURING MOUSE EYE DEVELOPMENT

Overview

It is well established that normal eye development requires complex morphological changes which are mediated through various signaling transduction pathways, including those modulated by the Rho family proteins which regulate cell differentiation and cytoskeletal changes. Currently, no data exists detailing the expression patterns of the three major Rho GTPases, RhoA, Rac1 and Cdc42, in the cornea and lens, nor has it yet been studied in the mammalian retina. Our results demonstrate both temporal and spatial differences in the expression patterns of all three GTPases in the cornea, lens and retina of the embryonic, neonatal and adult mouse eyes. Immunohistochemistry was used to examine the expression patterns of RhoA, Rac1 and Cdc42 in the embryonic (11.5 p.c., 14.5 p.c., and 17.5. p.c.), postnatal (one day), and adult C57BL/6 mouse eye. To confirm the expression pattern of these Rho GTPases, western blot and RT-PCR analysis was also conducted. Results showed differences in both spatial and temporal expression throughout the development of the eye and into the adult tissues. Immunohistochemistry revealed that all three Rho GTPases were expressed early in eye development, but that such expression was modulated during development differently within different ocular tissues. Differential expression was maintained in the adult expression, particularly within the lens, which was confirmed using both western and RT-PCR analysis. The GTPases are differentially

expressed in the cornea, lens and retina of mouse eye during development. The three small molecule GTPases are strongly expressed in the embryonic lens, with relatively less expression in the cornea and retina. In the neonatal mouse, all GTPases displayed their strongest expression within the retina. In the adult mouse eye, Rac1 but not RhoA nor Cdc42 were expressed during lens growth and secondary fiber cell differentiation. All three Rho GTPases are expressed during corneal epithelial cell differentiation, however, only RhoA is expressed during endothelial cell differentiation. In contrast, all three small GTPases are expressed in retina.

Introduction

Small molecule GTPases function as "molecular switches" mediating extracellular signaling events to downstream intracellular effectors by cycling between GDP-bound inactive and GTP-bound active forms upon stimulation. The Rho family of small molecule GTPases, of which RhoA, Rac1 and Cdc42 are most highly characterized, are implicated in numerous cellular functions including cytoskeletal actin organization (Ridley and Hall, 1992), cell growth, cell migration (Zipkin et al., 1997), cell adhesion (Ridley et al., 1992), vesicular trafficking, and differentiation (Hall, 1998). These different Rho family GTPases regulate the formation of distinct structural elements; Rho induces formation of actin stress fibers and cell contacts, whereas Cdc42 and Rac1 regulate filopodia formation and membrane ruffling, respectively (Hall, 1998). Although once thought to be ubiquitously and uniformly expressed in mammalian tissues, differential expression patterns of these proteins have been shown in the brain (O'kane et al., 2003), as well as throughout retinal development in the chicken (Santos-Bredariol et al., 2002). Differences in expression of

these Rho family proteins in neuronal cells has proven to be essential for proper neurite outgrowth, as each member prompts distinct effects on the growth of neuronal processes and axons, and it is possible that differential expression patterns of these proteins in other tissues is equally important for the growth and perpetuation of other cells and systems (Hall, 1998)

The vertebrate eye develops through a complex process of morphogenesis which is modulated by various signaling transduction pathways and governed by different families of regulators. Among these regulators are the small molecule GTPases. Using radioactive GTPase blot overlay assays on soluble and insoluble fractions of monkey and human lenses, Rao (1997) first demonstrated the presence of multiple proteins primarily within the insoluble membrane fractions which possessed strong GTP-binding abilities, including the small molecule GTPases which ranged in size between 20-30 kDa corresponding to the molecular weights of Rho and Rac. Blockage of Rho isoprenylation in cultured rabbit lens epithelial cells via lovastatin treatment inhibits Rho association with the membrane, resulting in the distortion of elongating epithelial cells and deterioration of the central epithelium due to the overall loss of focal adhesions, cell-cell adhesions, protein-phosphotyrosine and actin stress fiber degeneration (Rao et al., 1997a; Maddala et al., 2001b). Additionally, transgenic mice overexpressing the C3 exoenzyme, a bacterial toxin which inactivates Rho GTPase, showed dramatically altered cytoskeletal organization, and microarray analysis of transgenic lenses demonstrated aberrant expression of over forty different genes, including those encoding extracellular matrix and basement membrane proteins (Maddala et al., 2004). Using immunohistochemistry, the same group has

analyzed the developmental expression pattern of another small GTPase, RhoB, in mouse lens from different embryonic stages to postnatal development up to 18 days. It was determined that RhoB was strongly expressed in the developing lens, specifically paralleling the morphogenesis of the lens fiber cells. Such results suggest its important role in lens differentiation (Maddala et al., 2001b). Considering the likely role of RhoB in lens differentiation, the possible roles of other small molecule GTPases, such as RhoA, Rac1 and Cdc42 remained to be determined.

The retina is commonly regarded as an archetype of cellular differentiation because of its complex composition of highly differentiated cell types. Four members of the Rho family of small molecule GTPases, RhoA, RhoB, Rac1 and Cdc42, were previously found to be differentially expressed in developing chicken retina, however whether such differential expression patterns are maintained through adulthood has yet to be determined (Santos-Bredariol et al., 2002). Additionally, previous studies have shown indicated clear differences in RhoB expression patterns between the mouse and chick lens, but whether such differential patterns of expression exist for the other major small molecule GTPases, RhoA, Rac1 and Cdc42 is an important question that has not yet been addressed.

To further explore the potential functions of the small molecule GTPases in regulating the development of different compartments of the eye, we have examined the expression patterns of RhoA, Rac1 and Cdc42 throughout mouse eye development. Our results are the first to reveal the expression patterns of these small molecule GTPases in cornea, and of the temporal differences in expression patterns of RhoA, Rac1 and Cdc42 in the ocular lens from embryonic stages, neonatal to adult. In addition, our studies provide

comparative information on the expression patterns of RhoA, Rac1 and Cdc42 in developing retina as well as their expression patterns in the adult retina. Together, our studies provide important information regarding the functions of RhoA, Rac1 and Cdc42 in regulating morphogenesis of the three compartments of the eye: retina, lens and cornea.

Expression of RhoA, Rac1 and Cdc42 in the Embryonic Mouse Eye

By 11.5 p.c., the lens vesicle itself has formed, and the ectoderm lining it begins to differentiate into cornea (Fig. 23A). Compared with the control (the rightmost panel) expression of RhoA and Cdc42 was clearly detected in this tissue overlaying the lens vesicle (the leftmost panel and the third panel from the left of Fig. 23A). In contrast, expression of Rac1 was nearly undetectable (the second panel from the left of Fig. 23A). RhoA and Cdc42 continue to be expressed at 14.5 p.c., and by this stage of development, expression of Rac1 also begins to appear within the cornea (Fig. 23B). At 17.5 p.c., when the cornea differentiates into three different layers: endothelial cells, stroma and epithelial cells, expression of RhoA and Cdc42 was found more focused in the epithelial and endothelial cells (the leftmost panel and the third panel from the left of Fig. 23C), whereas, expression of Rac1 parallels this same pattern of expression but at a level significantly lower that RhoA and Cdc42 (the second panel from the left of Fig. 23A).

At 11.5 p.c., strong expression of the RhoA was detected throughout the lens vesicle, a pattern similar to that found with RhoB (Maddala et al., 2001a) (the most left panel of Fig. 23A). Expression of Cdc42 at this same stage was lower than that of RhoA, while Rac1 expression was barely detectable (the third and the second panels from the left

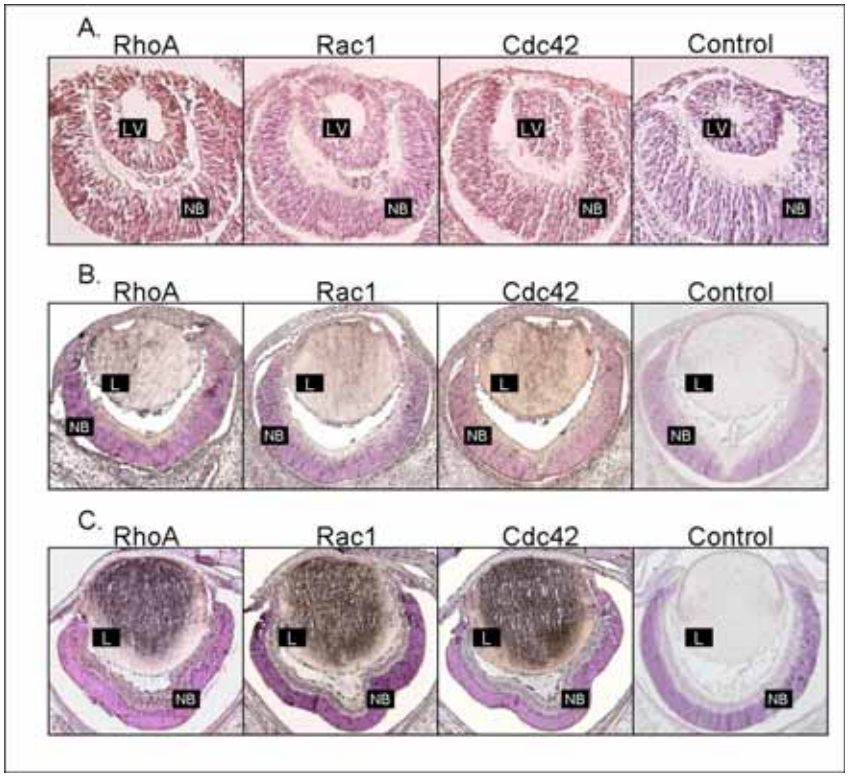

Figure 23: Expression of Rho GTPases during Embryonic Development. (A) Day 11.5 p.c. expression of RhoA, Rac1 and Cdc42 is present throughout the mesenchyme [M] and in the developing neuroepithelium [NE], particularly on the outer region adjacent to the lens vesicle [LV]. (B) By day 14.5 p.c., expression of the Rho family GTPases is elevated in the highly differentiating lens fibers [L] as well as in the inner region of the neuroblastic layer [NB] lining the optic cup. Expression of Rac1 is pronounced throughout the differentiating lens cells, whereas, RhoA and Cdc42 expression appears concentrated along the cuboidal layer of the lenticular cells. (C) Day 17.5 p.c. mouse eye shows Rho GTPase expression at the actively dividing layer of the lens, with moderate and diffused expression in the neuroblastic layer.

of Fig. 23A, respectively). In contrast to Rac1 expression, which was predominantly found in the differentiating primary fiber cells (the second panel of Fig. 23B), both RhoA and Cdc42 expression at 14.5 p.c., was found in differentiating primary fiber cells as well as in the lens epithelium and (the leftmost panel of Fig. 23B and the third lane from the left of Fig. 23B, respectively). Overall, expression of Cdc42 at 14.5 p.c. is relatively higher than either RhoA or Rac1 (Fig. 23B). All three small molecule GTPases are more strongly expressed by 17.5 p.c., displaying heavy expression in the differentiating primary fiber cells and in the subcortical region (Fig. 23C). Enhanced expression of all three Rho GTPases was found in the primary fiber cells, and increased to a lesser extent in the lens epithelium. In contrast, the expression of these small GTPases was much attenuated in the cortical region (Fig. 23C).

In contrast to the predominantly postnatal expression of RhoB, RhoA was found expressed at 11.5 p.c. in the lens vesicle and strongly expressed in the developing neuroretinal layers, the neuroblastic region (the leftmost panel of Fig. 23A) (Maddala et al., 2001a). During this same stage of development, the expression level of Cdc42 in retina was lower than RhoA, and that of Rac1 was lower still (the third and the second panels from the left of Fig. 23A, respectively). At stage 14.5 p.c., expression of RhoA was strongly detected in the predicted photoreceptor zone and to a less degree in the ganglion cell layers (the leftmost panel of Fig. 23B). Although Rac1 and Cdc41 reveal similar patterns of expression, the overall level of Rac1 seems higher than those of the other small molecule GTPases (Fig. 23B). As development proceeds to 17.5 p.c., expression of all three small molecule GTPases are detected in the photoreceptor layers (Fig. 23C).

Expression of RhoA, Rac1 and Cdc42 in the Neonatal Mouse Eye

Compared to the negative control (Fig. 24D), RhoA was expressed in the endothelium and to a lesser degree in the epithelial cells and stroma of the neonatal mouse (Fig. 24A). Similarly, expression of Cdc42 was clearly detected in both endothelial and epithelial cells, but hardly detectable in the stroma (Fig. 24C). In contrast to both RhoA and Cdc42, strong levels of Rac1 expression were noted in the fibroblasts within the stroma as well as in both endothelial and epithelial cell layers of the neonatal mouse cornea (Fig. 24B). In the ocular lens, strong expression levels of RhoA, Rac1 and Cdc42 were detected in the lens epithelial cells (Fig. 24A, 24B and 24C) of the neonatal mouse in comparison to the negative control (Fig. 24D). In contrast, their expression levels in the fiber cells were substantially decreased (Fig. 24A, 24B and 24C).

Compared with the negative control (Fig. 24D), a strong expression level of RhoA was detected in the photoreceptors, the horizontal/amacrine/Muller's cells, as well as some of ganglion cells of the neonatal mouse retina (Fig. 24A). In most of the ganglion cells, inner and outer plexiform layers, a reduced level of RhoA was detected (Fig. 24A). Expression of Rac1 and Cdc42 followed a similar pattern of RhoA but the expression levels were decreased in the retina of neonatal mouse eye (Fig. 24B and 24C).

Expression of RhoA, Rac1 and Cdc42 in the Adult Mouse Eye

Contrasting the expression patterns of RhoA and Cdc42 in the cornea of the adult mouse eye, immunocytochemistry revealed that both RhoA and Cdc42 were absent in the ocular lens, neither in the epithelial cells nor in the fiber cells (Fig. 25; leftmost and rightmost panels). Rac1 expression, however, was found throughout the lens, exhibiting a

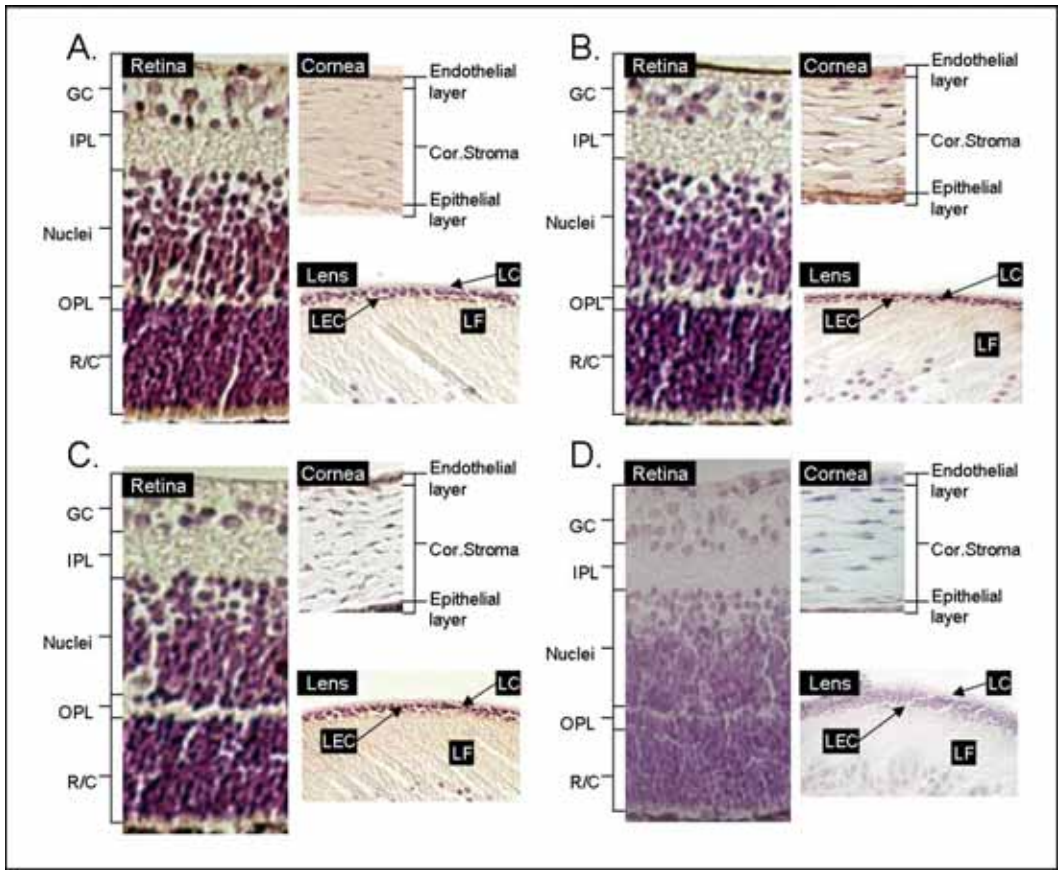

Figure 24: Expression of Rho GTPases in Neonatal Mouse Eye. (A) RhoA was expressed diffusely throughout the anuclear regions of the retina at day1. Corneal expression of RhoA was elevated throughout the tissue, with enhanced staining at the endothelium. RhoA staining in the lens fibers [LF], however, higher expression was seen in the lens epithelial cells [LEC] and lens capsule [LC]. (B) Expression of Rac1 was only slightly detectable in the retina, but more intense staining was seen in the corneal endothelium as well as in the LF, particularly concentrated in the LEC. (C) Cdc42 staining was weak in the retina, corneal stroma, and LF. Expression of Cdc42 was enhanced in the corneal endothelium and the LEC.

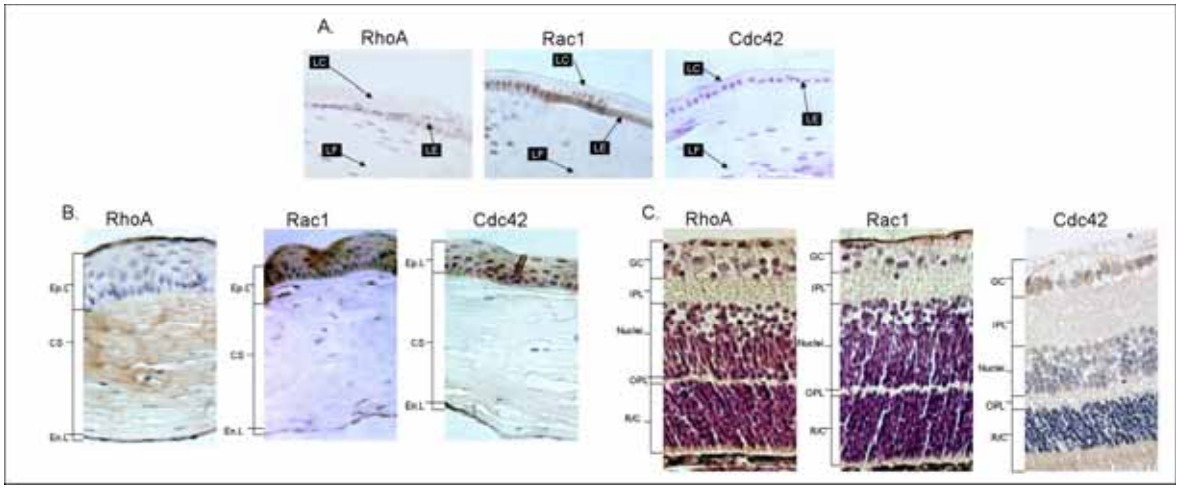

Figure 25: Expression of Rho GTPases in Adult Mouse Eye. (A) IHC of the adult lens shows RhoA expression is undetectable. Lens fibers [LF] showed diffuse Rac1 expression throughout the adult lens. No detectable Cdc42-specific staining was seen in the adult mouse lens. (B) RhoA expression in the cornea shows high immunoreactivity in the regions of the columnar and squamous cells, as well as elevated in the posterior epithelial layer [EpL]. IHC of the adult mouse cornea exhibited elevated expression of Rac1 in the columnar and squamous cell regions and moderate staining in the posterior endothelium [EnL]; no discernable staining was seen in the corneal stroma. Cdc42 expression was primarily isolated to the cornea with a small amount of diffuse staining in the posterior endothelium. (C) The adult retina showed RhoA staining in every region. The staining was strongest in the photoreceptor cell region. Rac1 was expressed primarily in the photosensitive cells, with lesser staining seen outer and inner plexiform layer [IPL] as well as in the ganglion cell [GC] layer. In the retina, elevated expression of Cdc42 was found in photoreceptor cells, with less staining in the GC and plexiform cell layers.

strong level of expression in the epithelial cells and less in the fiber cells (Fig 25; center panel). The absence of RhoA and Cdc42 in the ocular lens of the adult mouse eye was further confirmed by Western blot analysis (Fig. 26).

Immunohistochemistry revealed that the highest level of RhoA expression in the cornea of the adult mouse eye was detected in the endothelial cells and the surface layer of epithelial cells (Fig. 25B; leftmost panel) with little detectable staining in the columnar cells of the corneal epithelium. In the corneal stroma, diffuse staining was detected in some fibroblasts (Fig. 25B; leftmost panel). In contrast, Rac1 was predominantly expressed in the entire layers of corneal epithelium (both columnar and squamous cell layers) but absent in both the corneal stroma and in the endothelial cells layer (Fig. 25B; center panel). For Cdc42, immunocytochemistry demonstrated that it was expressed in both the endothelial cell layers and the entire layers of corneal epithelium (both columnar and squamous cell layers) but not in the corneal stroma (Fig. 25B; rightmost panel). To further confirm the expression of RhoA, Rac1 and Cdc42 in the cornea of the adult mouse eye, western blot analysis was conducted. As shown in Fig. 26, all three small molecule GTPases were detected in the cornea of the adult mouse eye.

In the retina of the adult mouse eye, immunocytochemistry demonstrated that both RhoA and Cdc42 were highly expressed in the photoreceptors, in the horizontal/amacrine/Muller cell layers as well as in some of the ganglion cells (Fig. 25C; leftmost and rightmost panels). In most ganglion cells, the inner and outer plexiform layers, expression of RhoA and Cdc42 was detectable but in a much reduced level (Fig. 25C; leftmost and rightmost panels). For Rac1, a much lower level of expression was

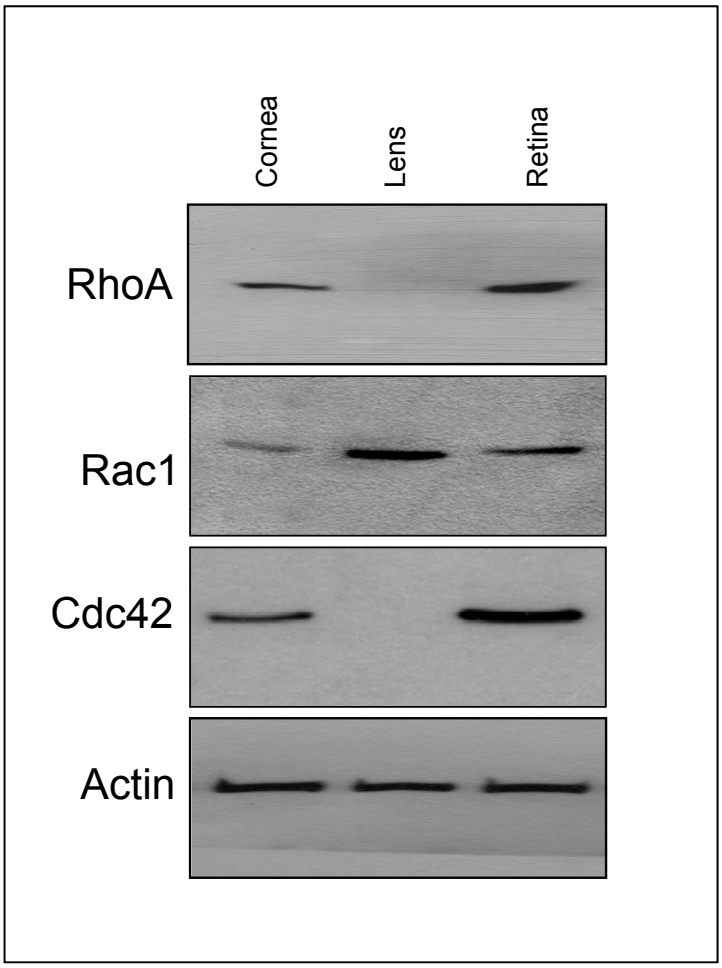

Figure 26: Western Blot Analysis of Adult Eye Tissues Corresponds to IHC Expression. Western immunoblot of mouse eye fractions showed significant expression of RhoA in the cornea and retina, with no discernable expression in the lens. Rac1 was expressed highly in the lens and retina, as well as moderately in the cornea. Expression of Cdc42 was found to be moderate in the retina and lens using western blot immunoassay.

detected in the retina of the adult mouse eye (Fig. 25C; center panel). The relative expression levels of RhoA, Rac1 and Cdc42 in the adult mouse retina was further confirmed by Western blot analysis (Fig. 26).

Rho Family GTPase Distribution during Ocular Development

Immunohistochemical analysis of Rho GTPase expression revealed that RhoA, Rac1 and Cdc42 are expressed early in mouse ocular development. Already by day 11.5 p.c., just as the lens vesicle is formed, expression of the Rho GTPases can be detected in the neuroblastic region, as well as throughout the lens cells. At day 14.5, just after the nerve fibers appear and grow towards the optic pit to form the optic stalk, but before they are completely differentiated, Rac1 expression becomes significantly elevated in the lens and lens fibers. Expression of Rho members is mostly uniform throughout the retina at this stage, with slightly higher expression of Cdc42 in the ganglion cell region. By day 17.5 p.c. Rho GTPase expression is concentrated at the outer, actively dividing periphery of the lens. Expression of RhoA, Rac1, and Cdc42 is maintained in neuroblastic regions, especially in the inner, nuclear portion of neuroblastic epithelium.

In the adult mouse eye, expression patterns of RhoA, Rac1 and Cdc42 continue to be distinct to different regions. RhoA appears to be most highly expressed throughout the cornea, particularly in the squamous cells and posterior endothelium with diffused staining in the columnar cell layer. RhoA in the retina is also increased in the photosensitive cells. Only slight expression of Cdc42 was seen in either the adult mouse cornea or retina, with the highest staining in the columnar cells of the cornea. Finally, Rac1 was the only GTPase detected in the adult lens, however high Rac1 expression was also seen in the

cornea within the columnar and squamous cell layers and in the retina with diffuse expression in the photosensitive cells, ganglion cells and both inner and outer portions of the plexiform layer.

Rho Family GTPases are Important for Eye Development and Maintenance

Previous studies have shown that the Rho family of GTPases is essential in modulating cytoskeletal rearrangements as well as mediating adhesive and morphological properties of the cell, transcriptional regulation and differentiation (Hill et al., 1995; Van Aelst and D'Souza-Schorey, 1997). For that reason, it was not surprising that RhoA, Rac1 and Cdc42 are all highly expressed during mouse ocular tissue development and continue to be specifically expressed in various regions of the adult eye. Although RhoA is known to function in opposition to Rac1 and Cdc42-mediated activity in many cell systems, it is likely that the presence of each is essential during eye development. Our expression analysis of the adult mouse showed that each of these Rho GTPases was isolated to discrete regions of the adult eye, suggesting that their presence is necessary for the maintenance and normal function of these areas. It is likely that during retinal development Rho GTPases play an essential role in regulating neuritogenesis and controlling neural cell growth, differentiation, guidance and branching as they have been found to regulate similar functions in Xenopus, Drosophila and mammals.

Elevated expression of the GTPases, particularly RhoA suggests a crucial role for these proteins in modulating eye development. Additionally, as Rac1 appears to be singly expressed at high levels in the adult mouse lens, changes in Rac1 expression and may be associated with the development of lens pathologies, such as cataract development and age-related structural changes. Indeed, treatment of cultured lenses with lovastatin shuts down Rho signaling-induced cataract formation, suggesting that activity of the Rho GTPases is essential in maintaining normal lens morphology (Rao et al., 1997b; Maddala et al., 2001b). In conclusion, our studies that elevated expression pattern of RhoA, Rac1 and Cdc42 in the developing eye reveals that Rho family of GTPases are potentially very important during embryonic development and ocular tissue differentiation. Differing patterns in distribution of each of these proteins in the adult suggests unique roles for the Rho family members in normal eye function and maintenance.

Discussion

Immunohistochemical analysis of Rho GTPase expression revealed that RhoA, Rac1 and Cdc42 are expressed early in mouse ocular development. Already by day 11.5 p.c., just as the lens vesicle is formed, expression of the Rho GTPases can be detected in the neuroblastic region, as well as throughout the lens cells. At day 14.5, just after the nerve fibers appear and grow towards the optic pit to form the optic stalk, but before they are completely differentiated, Rac1 expression becomes significantly elevated in the lens and lens fibers. Expression of Rho members is mostly uniform throughout the retina at this stage, with slightly higher expression of Cdc42 in the ganglion cell region. By day 17.5 p.c. Rho GTPase expression is concentrated at the outer, actively dividing periphery of the lens. Expression of RhoA, Rac1, and Cdc42 is maintained in neuroblastic regions, especially in the inner, nuclear portion of neuroblastic epithelium.

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CHAPTER VII

RHO FAMILY GTPASE REGULATION OF LENS EPITHELIAL DIFFERENTIATION

Overview

Development and differentiation of lens tissue require precise regulation of cellular pathways regulating the unique structural organization which affords the lens its transparency and ability to focus light. Although structurally simple and composed of a single metabolically active layer of epithelial cells, any anomaly in normal function and maintenance can lead to a multitude of pathologies including cataractogenesis, presbyopia and blindness. Western blot analysis in conjunction with immunohistochemistry has indicated a possible correlation between heightened Rac1 expression and increased levels of lens differentiation. In vitro data using lens epithelial cell culture has identified a direct relation between active levels of Rac1 and higher expression of the lens cell differentiation marker, β-crystallin. Luciferase assays indicate modulation of crystallin promoter activation upon activation of Rac1 using GEFT, a guanine nucleotide exchange factor expressed in the lens. Additionally, analysis of the promoter region of αB-crystallin has revealed the presence of multiple putative SRF sites, a direct downstream effector of activated Rac1. Here we show that SRF modulates lens cell differentiation through its regulation of crystallin transactivation via activation of Rac1 small molecule GTPase.

Introduction

Nearly one in every six Americans over the age of 40 is affected by cataracts, a statistic that involves over 20 million in the United States alone; worldwide, the number

would staggering. Thus great efforts are being made to understand the development and maintenance of the normal lens in order to better treat lenticular defects and opacification. The lens consists of a single actively dividing epithelial layer. As new lens epithelial cells form, older cells will migrate posteriorly, become elongate and gradually differentiate into lens fibers. These lens fibers, although anuclear and quiescent in nature, are structurally highly differentiated. Lens differentiation is initiated and controlled by numerous growth factors, including TGF-β, fibroblast growth factor (FGF) and bone morphogenetic proteins which are found in the vitreous humor (Zelenka, 2004). These growth factors then modulate cytoskeletal alterations that transform the cuboidal lens epithelial cell shape into the elongate-shape characteristic of the lens fiber. Additionally. the migration of differentiating epithelial cells away from the metabolically active region requires coordination of cell-cell and cell-matrix interactions. Such cytoskeletal alterations, cell migration and adhesion necessary for normal lens differentiation are all functions regulated by the Rho family of small molecule GTPases (Zelenka, 2004). Consequently, there has recently been a growing interest in determining the function of the Rho family in lens development and differentiation, as well as in the possible role the Rho family has in modulating lens pathologies, particularly opacification leading to cataractogenesis.

Genes encoding membrane and cytoskeletal proteins, as well as the lens crystallins have all been found to contribute to the formation of cataracts. Lens crystallin proteins consist of three major classes, α, β and γ, all having different functions within the lens. Alpha-crystallins are particularly important as they are the proteins which maintain lens transparency. Alpha crystallins act as molecular chaperones and aberrations in their

function and in their expression lead to abnormal protein aggregation that can cause to opacification (Zelenka, 2004). The following research demonstrates that the crystallin promoter activation is under the control of the Rho family of small molecule GTPases, specifically Rac1. Immunohistochemistry and western blot analysis show that the highest levels of Rac1 expression are reached during periods of high lens epithelial cell differentiation and this consequently leads in Rac1 mediated activation and nuclear localization of SRF which transactivates crystallin expression.

Differential Expression of Rac1 in the Lens

Historically, the Rho family of small molecule GTPases are considered to be uniformly expressed throughout tissues. New studies, however, suggest that unique temporal and spatial expression of the Rho family in areas such as the brain are essential for normal development and cellular differentiation. Initially, our lab sought to determine whether there was differential expression of the major Rho family members: RhoA, Rac1 and Cdc42 within the various tissues of the eye. Western blot analysis of corneal, lenticular and retinal tissues revealed that unlike RhoA and Cdc42, Rac1 was highly expressed in the adult mouse lens tissue (Figure 27A). Additionally, immunoblot of lens tissues from young, adult and aged mice showed increased Rac1 expression in the highly differentiating young mouse lenses (~2 weeks) and in the aged mouse lenses (>1 year) (Figure 27B). These results suggest that unique Rac1 expression in the eye is both spatial (expressed highly in the lens) and temporal (expressed at different times of development).

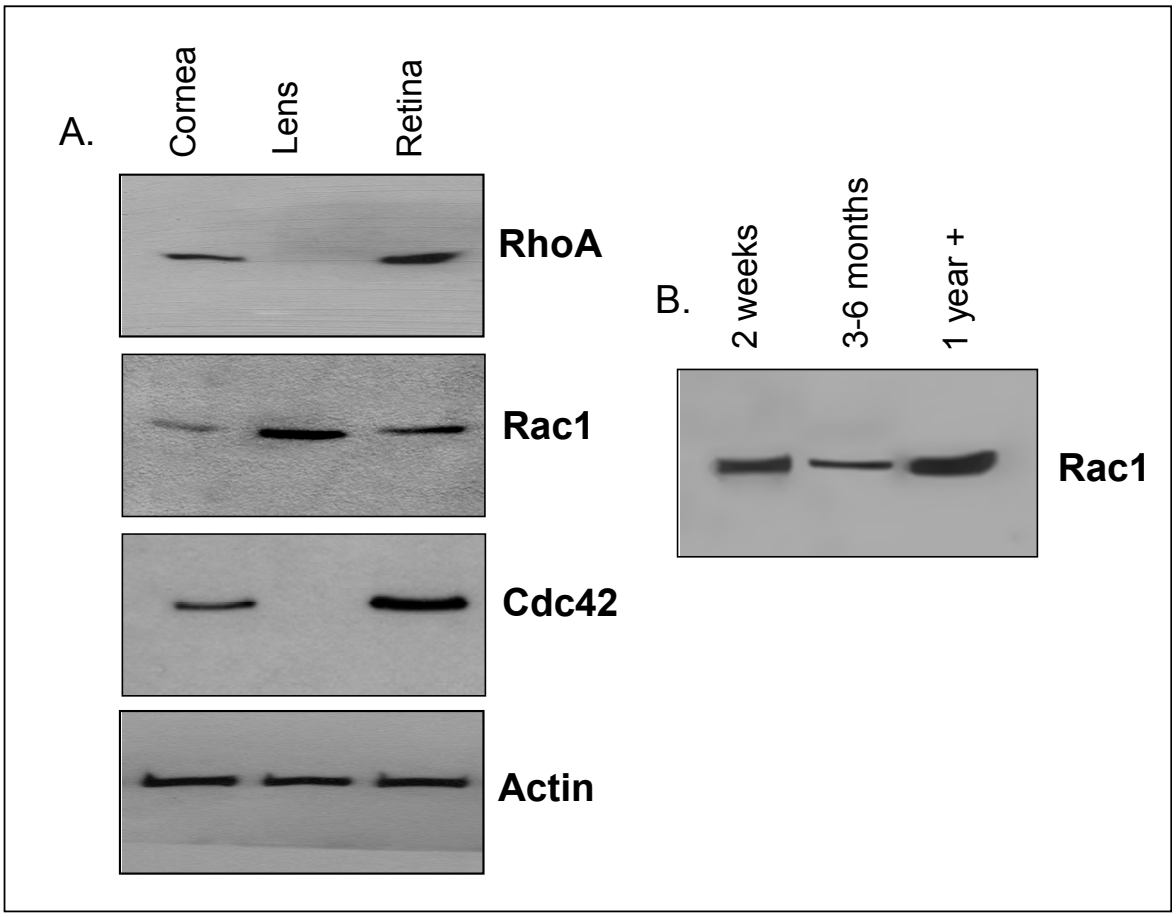

Figure 27: Unique Spatial and Temporal Expression of Rac1 in the Lens.

In addition, Rac1 was found to be expressed during periods of heightened lenticular differentiation: early, when lens cell differentiation is high, and in older mice when such differentiation might indicate aberrant cellular function and the development of lens pathologies.

Process of Lens Dell Differentiation Leads to Heightened Levels of Activated Rac1

To understand the potential role of Rac1 is lens differentiation, we used N/N003A rabbit lens epithelial cell line. As N/N003A cells become confluent, they become more differentiated and take on more characteristics of lens fibers, including more elongate shape and increased expression of β-crystallin, a marker of lens differentiation. Levels of activation were measured at three timepoints: 70% confluency, 100% confluency and 100% confluency after three days. Using activated-GTPase pull-down assays, we found that while total Rac1 levels increase only slightly (Figure 28), activated levels of the protein increase more dramatically. Conversely, RhoA activation decreased upon increased confluency and Cdc42 saw a slight increase both in total levels and in activated levels of the protein.

Activator of Rho family GTPases is Expressed Throughout the Eye

In order to determine whether activation of Rac1 was essential for Rac1 mediated lens differentiation, we used a guanine nucleotide exchange factor, GEFT, that regulates activation of the Rho family members, including Rac1, by inducing Rac1 dissociation from its inactive GDP-bound form to its active GTP-bound form (Figure 29A). To show that GEFT activation was physiologically relevant to Rac1 activation in the lens, we initially carried out western blot analysis of different fractions of the eye using a GEFT-specific

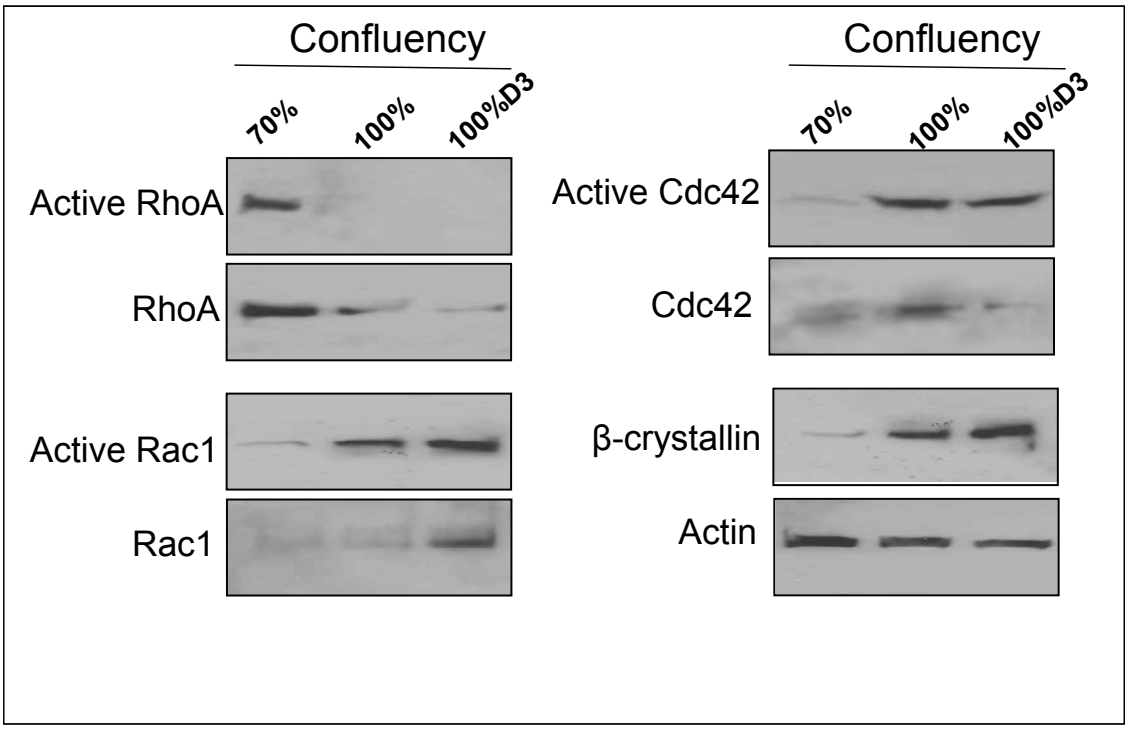

Figure 28: Differential Expression of Activated Rac1 in Lens Cell Culture.

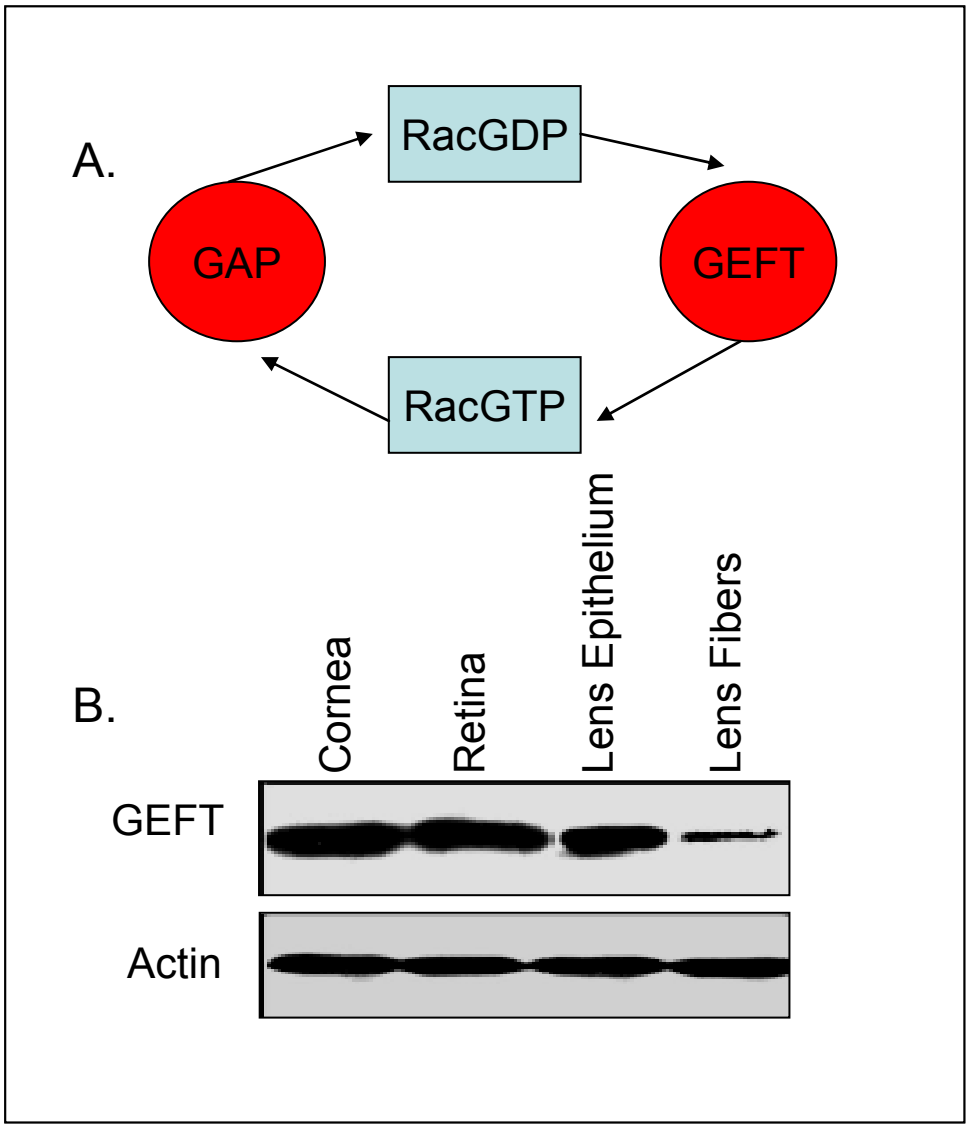

Figure 29: Rac1 Activator, GEFT, Expressed in the Lens.

antibody (Figure 29B). Results showed that GEFT was ubiquitously found throughout the different regions of the eye, including the lens fibers and lens epithelium. These results suggested that using GEFT-AAV infection of N/N003A cells would be a credible means of testing effects of Rac1 activation in lens culture.

GEFT Infection Increases Active Levels of Rho Family GTPases

N/N003A cells were infected with an AAV virus carrying a human isoform of GEFT. Activated-GTPase pull-downs using GST-tagged Pak and Rhotekin beads, was then used to determine activation of Rac1, Cdc42 and RhoA in cell culture. Results showed that whereas total levels of Rho family proteins was unaltered, the active form of all three Rho GTPases was significantly enhanced in the GEFT-infected cells (G) as compared to the vector-only transfected lens cells (V) (Figure 30). This data supports that while GEFT does not affect total levels of Rho family of protein, it does induce activation of all three major members in lens cell culture.

Lens Fiber Differentiation Ablated by Rac1 Specific Inhibitor

Inhibition of Rac1 in cell culture was mediated through use of the Rac1 specific chemical inhibitor, NSC23766. This inhibitor functions by interfering with Rac1 interaction with two major GEF proteins, Trio and Tiam1. To determine whether this Rac1 inhibitor would interrupt GEFT-Rac1 interaction, immunoblot and lens cell confluency analysis was used to determine degree of lens differentiation upon treatment. N/1003A cells were grown at 70% confluency and 100% confluency for 3 days; cells were untreated, GEFT-AAV infected, treated with 50 μM Rac1 inhibitor, or infected with GEFT-AAV and treated with Rac1 inhibitor (Figure 31). Results show that GEFT-AAV

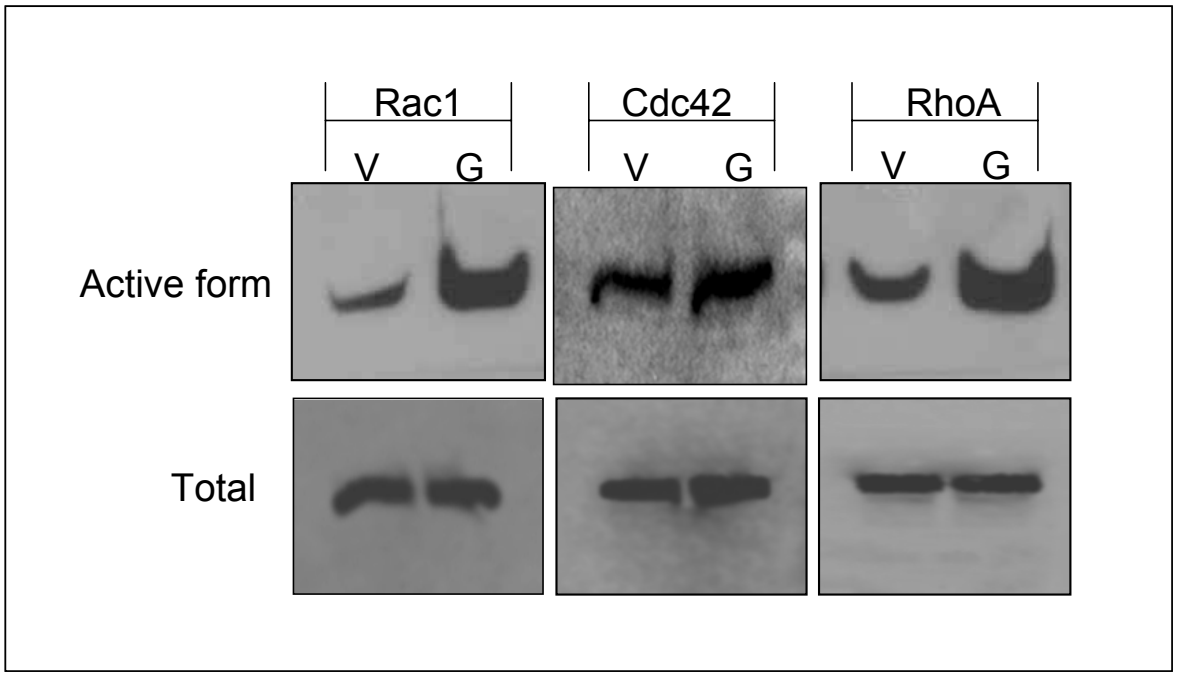

Figure 30: GEFT Infection Increases Active Rho GTPases

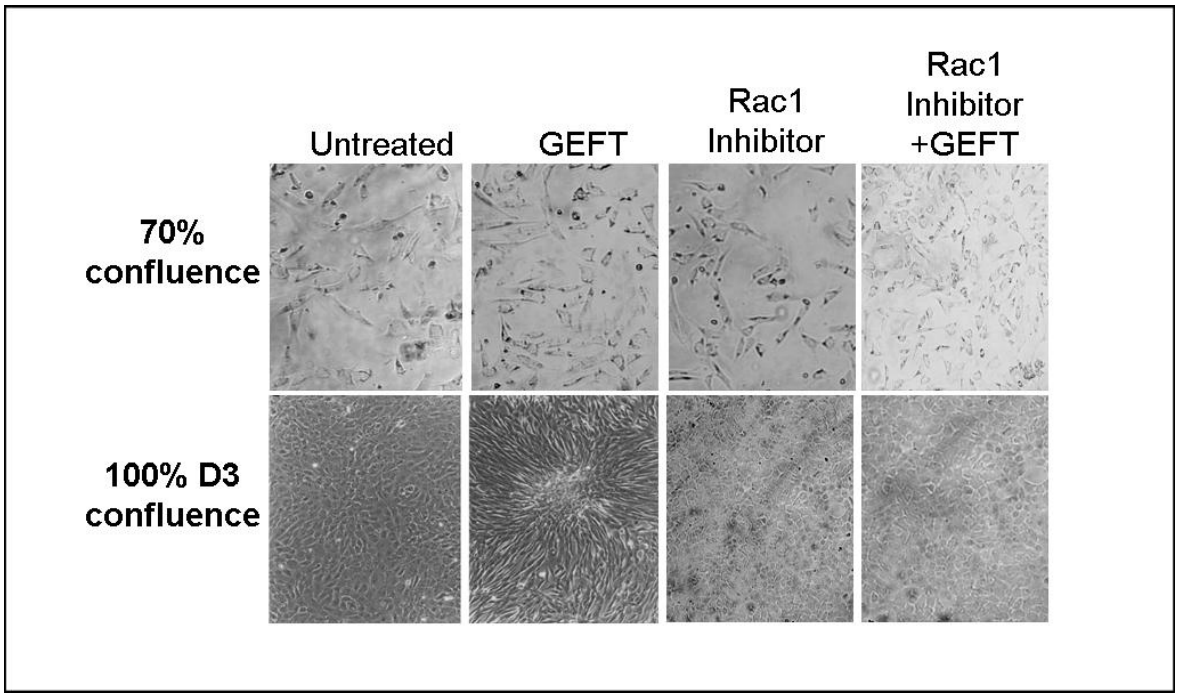

Figure 31: Lens Differentiation Ablated by Rac1 Inhibitor

infection induces high levels of cellular elongation characteristic of lens differentiation into lens fibers as compared to untreated. In addition, cells which were treated with the Rac1 inhibitor showed no significant morphological differences as compared to the untreated. However, treatment of GEFT-AAV infected cells with the Rac1 inhibitor ablated elongate cell morphology changes. Additionally, western blot analysis revealed that whereas GEFT infection induced high expression of the lens differentiation marker, β-crystallin, this effect was abolished upon treatment with Rac1 inhibitor (Figure 32). These results support Rac1 activation as a key mediator of lenticular differentiation.

Rho Family GTPase Regulation of Crystallin Expression

Crystallin expression is a marker for lens cell differentiation. As such, our lab attained luciferase constructs containing the promoter regions for six different crystallin proteins (two α-, two β- and two γ- crystallins). These constructs were transfected into N/N1003A cells along with RhoA, Rac1 and Cdc42, GEFT and the dominant negative forms of the Rho family members (Figure 33). Results showed that activation of the crystallin promoter (representative luciferase shown here) was attained only upon co-transfection of Rac1 with GEFT and this transactivation was ablated upon overexpression of the dominant negative form of Rac1, Rac1 T17N. Additionally, treatment of crystallin promoter-transfected N/N1003A cells using inhibitors to major signaling pathways, including inhibitors of JNK, Gαq, Rho kinase, p38, RhoA and the Rho GTPases revealed that inhibition of the Rho family using Toxin A led to inhibition of basal crystallin expression. These results showed that while Toxin A ablated basal crystallin transactivation, inhibition

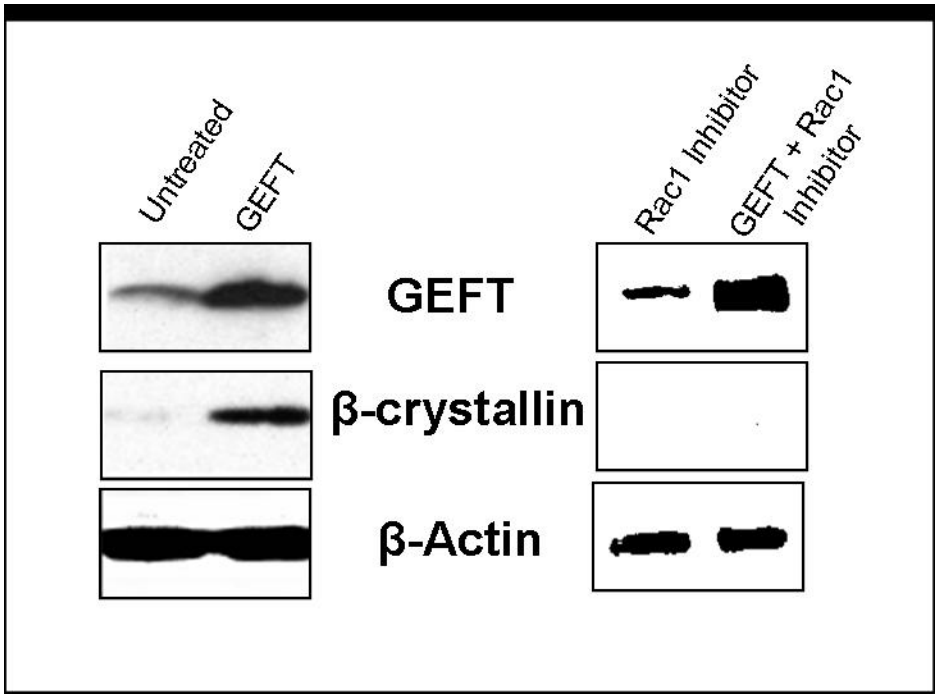

Figure 32: Rac1 Inhibitor Prevents β-Crystallin Expression.

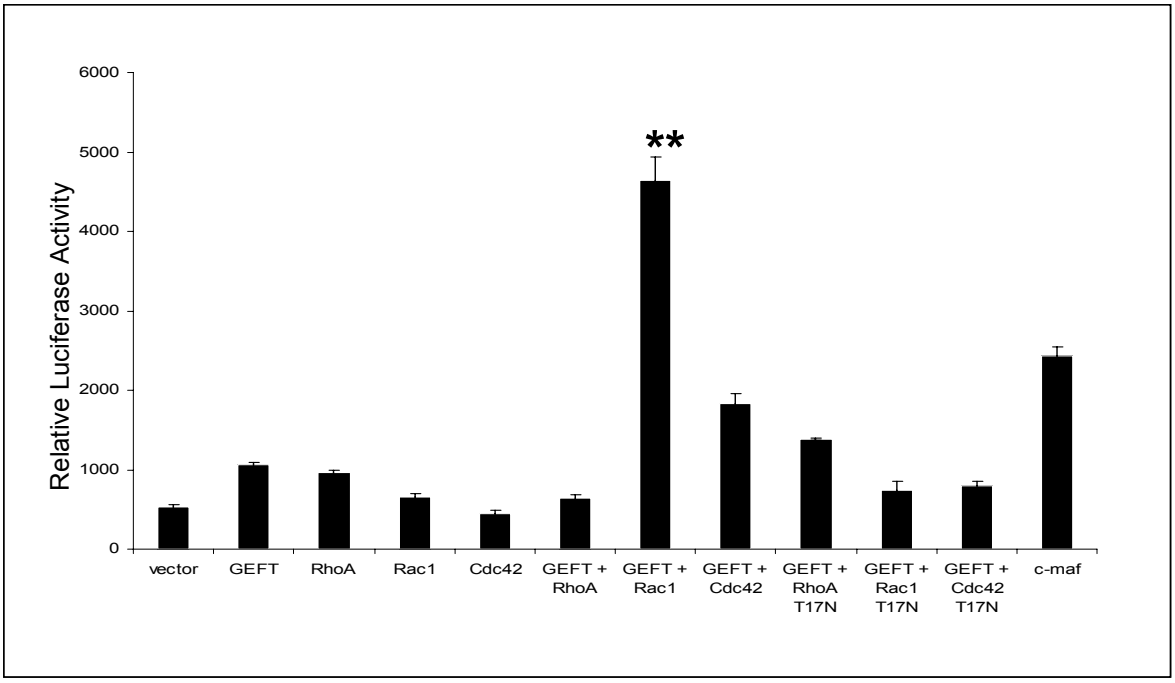

Figure 33: GEFT-Activated Rac1 Regulation of Crystallin Expression.

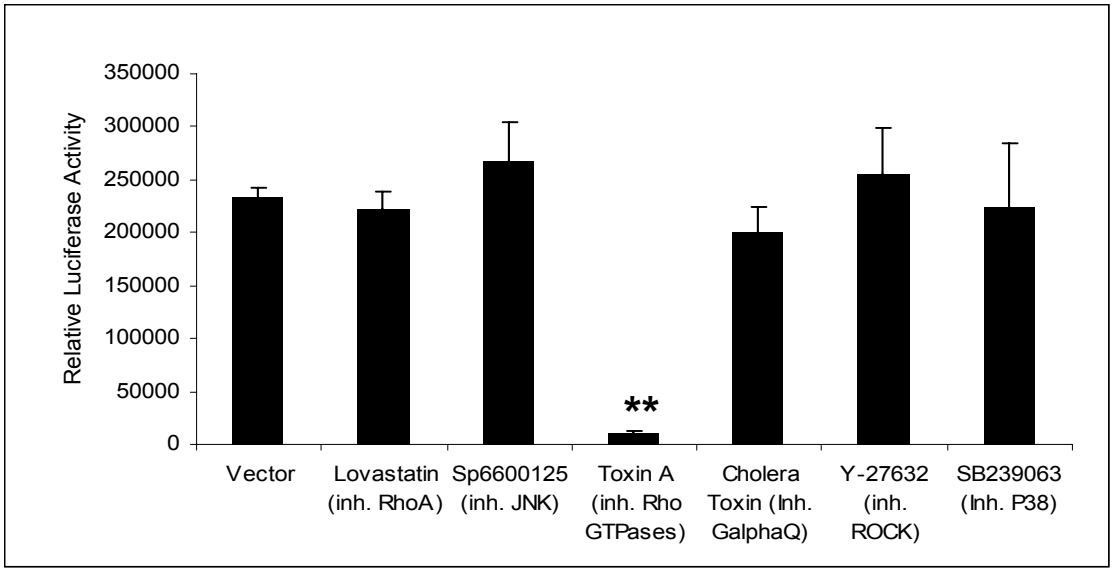

Figure 34: Rho GTPase Inhibitor, Toxin A, Obliterates Crystallin Transactivation.

of RhoA using Lovastatin did not inhibit crystallin promoter activation, suggesting a GTPase other than RhoA was responsible for the effect (Figure 34).

SRF Responsible for Mediating Lenticular Differentiation via Rac1 Activation

To determine the underlying signaling pathways modulated by activated Rac1 leading to lens differentiation, western blot analysis on the major downstream effectors was done using lysates from lens cells (Figure 35). The MAPK signaling pathways is the major signaling intermediary of Rac1 effects, however, our data showed no phosphorylation of ERK, p38 or JNK or significant changes in expression of total protein levels (Figure 35). Another major mediator of Rac1 effects is the serum response factor (SRF). To determine if SRF played a role in modulating lens differentiation via Rac1 activation, SRF was co-transfected along with Rac1 and GEFT in N/N1003A cells expressing αB-crystallin promoter (Figure 36). Although SRF increased GEFT and Rac1 mediated induction of αB-crystallin promoter activation, a SRF mutant lacking the c-terminal nuclear localization sequence, SRFΔ diminished αB-crystallin transactivation to basal levels. These results indicate the necessity of SRF action in the nucleus, likely as a transcription factor.

Activation of Rac1 in Lens Epithelial Cells Induces SRF Nuclear Localization

Considering the importance of the SRF nuclear localization sequence in inducing αB-crystallin transcriptional activation, immunofluorescence of GEFT infected cells was used to determine if Rac1 activation by GEFT induced nuclear localization of SRF (Figure 37). N/N1003A cells on glass coverslips were transfected with GEFT-flag and SRF-HA or Vector with SRF-HA, fixed and incubated with the appropriate antibodies. Results

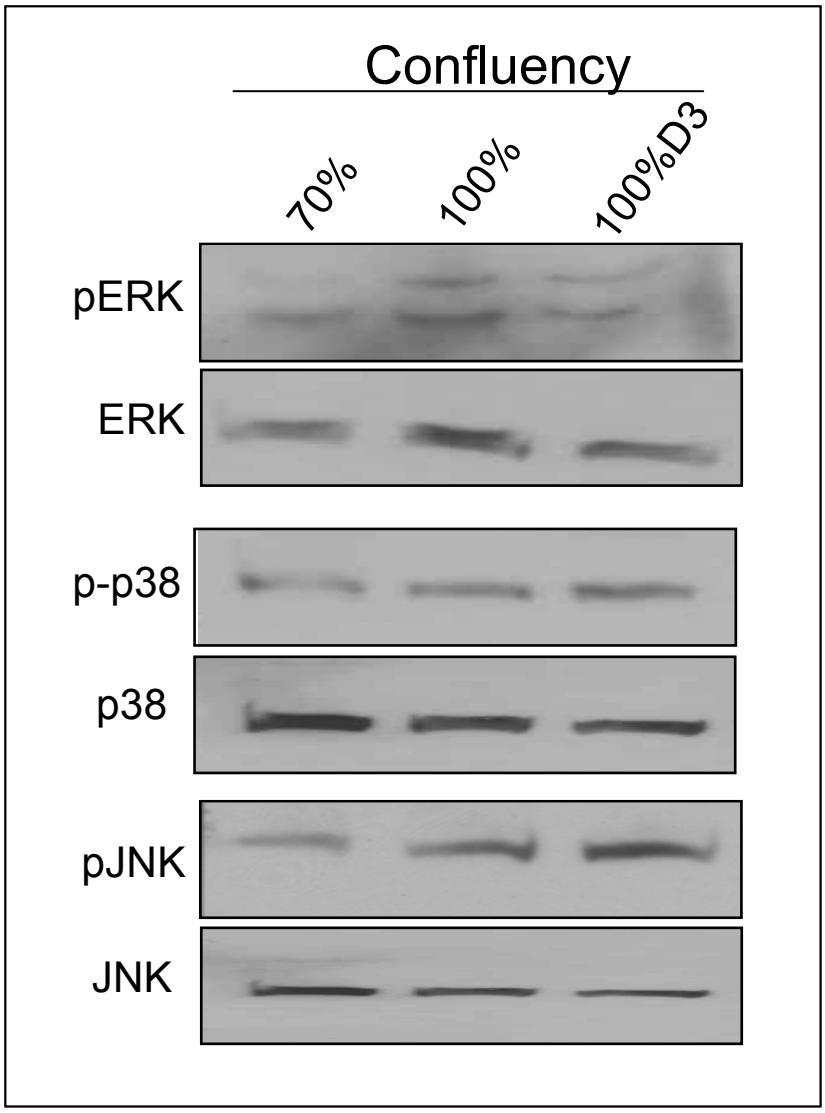

Figure 35: Lens Differentiation Not Dependent on MAPK Pathway.

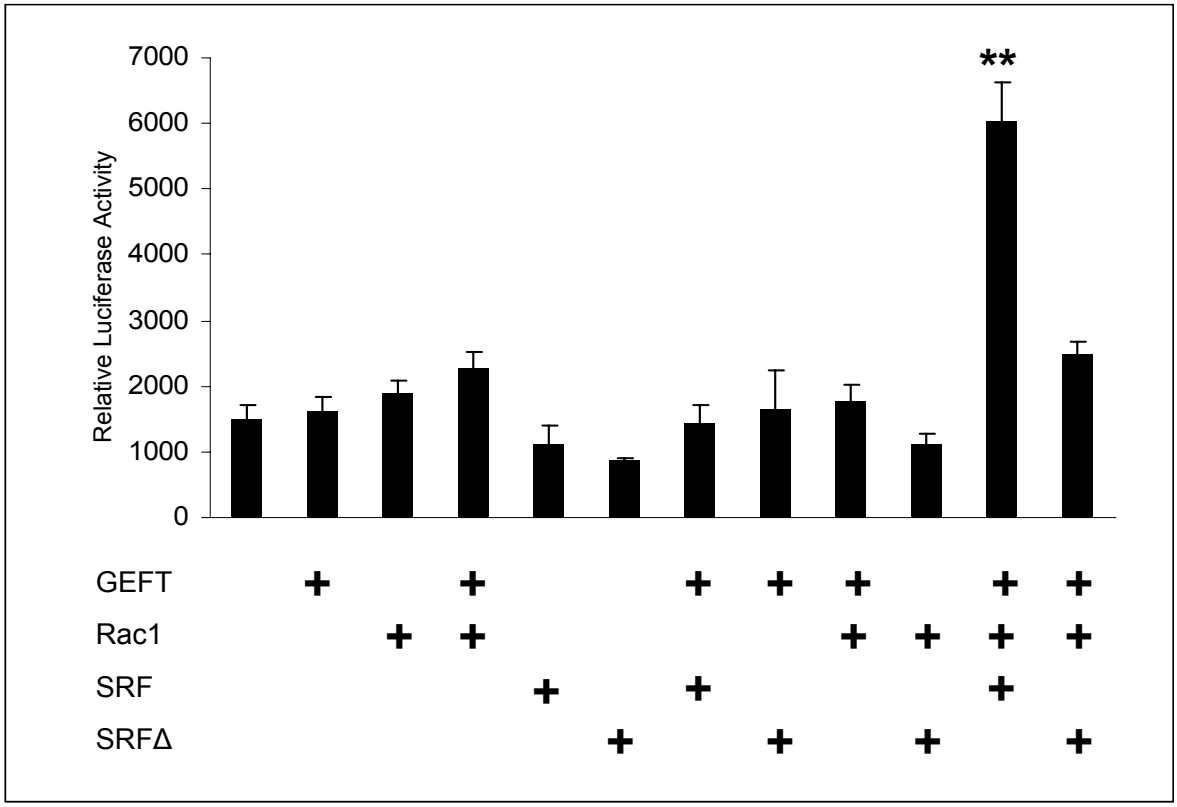

Figure 36: SRF Mediates Lens Differentiation via Rac1 Activation.

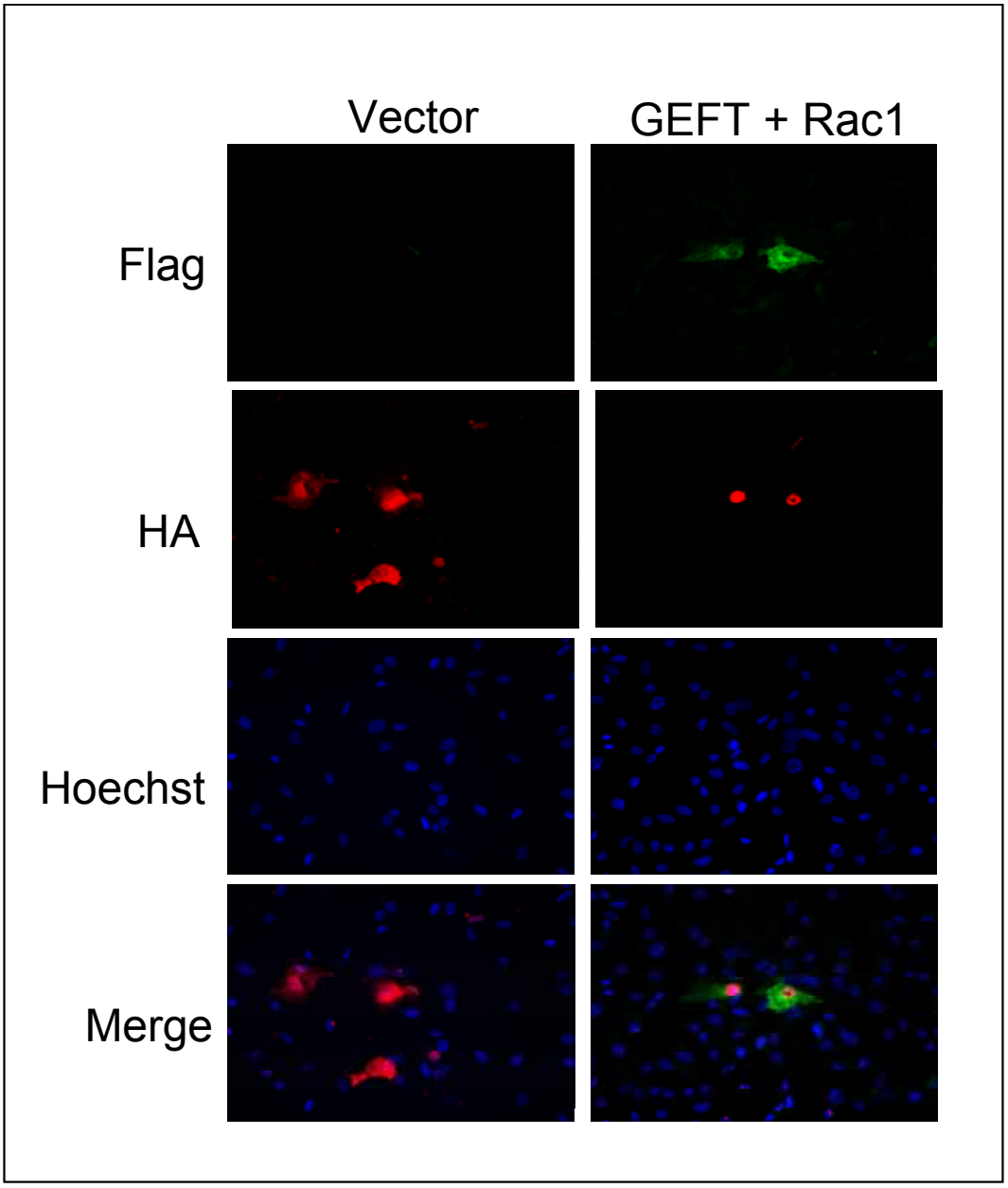

Figure 37: Rac1 Activation Induces SRF Nuclear Localization.

showed that SRF-HA was localized to the Hoechst-stained nuclei in the GEFT+Rac1 transfected cells, whereas no such nuclear localization was seen in vector-only transfected cells, suggesting activation of Rac1 may induce translocation of SRF to the nucleus.

Discussion

Serum response factor (SRF) is a mammalian transcription factor which is capable of acting as either a homodimer or by binding numerous other proteins to modulate transcription SRF most notably controls regulation of immediate early genes such as egr-1 and c-fos, as well as genes involved in myogenesis and is not surprisingly largely controlled through Rho mediated pathways (Carnac et al., 1998; Montaner et al., 1999; Psichari et al., 2002; Schratt et al., 2002). Aberrant SRF-mediated transactivation has previously been associated with pathologies of the vascular smooth muscle as well as smooth muscles of the respiratory system (Affolter et al., 1994; Guillemin et al., 1996; Lu et al., 1998; Ding et al., 2001). In addition, SRF is highly expressed throughout early developmental stages in the region of the eye, and its targeted disruption is lethal during the embryonic stages of mouse development suggesting it is a likely mediator of early lens differentiation (Arsenian et al., 1998; Weinhold et al., 2000; Wiebel et al., 2002)

Thus far, our results indicate a possible molecular mechanism underlying lenticular differentiation by which activated Rac1 in the lens induces SRF nuclear localization which induces transcriptional activation of the crystallin genes. We are currently determining whether putative SRF responsive elements (SREs) within the αB-crystallin promoter, and the promoters of numerous other crystallins directly mediates transcriptional regulation. Additionally, lens explant culture of whole lens epithelial layers harvested and cultured in

Rac1 inhibitor is resistant to TGF-β-induced lens differentiation and opacification. If these results are positive, this study will show a novel mechanism regulating lens differentiation and crystallin control via activated Rac1 effect on SRF localization.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

The majority of my Ph.D. research focused on understanding different aspects of the human metastasis suppressor peptide, KiSS-1: the transcriptional mechanisms by which it is regulated, identification of signaling intermediaries downstream of its cognate G-protein coupled receptor, and the means by which it becomes lost during metastatic progression. As the anti-metastatic effects of KiSS-1 register through activation of its membrane receptor, I began by developing a yeast-two-hybrid system in which the intercellular region of the receptor, including a SH3 binding domain, was used as bait to look for direct protein interaction. This analysis yielded multiple candidate proteins which were subsequently tested and verified using GST-pulldowns and cell culture-based co-immunoprecipitation assays. Results indicated direct binding of the KiSS-1 receptor with at least two SH3 domain-containing proteins, PSGAP (PH and SH3 domain containing Rho GTPase activating protein) and the guanine nucleotide exchange factor, Vav1. These findings are significant as they provide a mechanism whereby KiSS-1 regulates control of the Rho family of small molecule GTPases, a family of proteins known to regulate cytoskeletal re-organization, and may thus explain the basis of KiSS-1's anti-metastatic effects as it alters signaling to the actin-based cytoskeleton to reduce migration and invasion. Additionally, my research helped support the role of the MAPK pathway in registering the intercellular effects of KiSS-1. Treatment of metastatic breast cancer cells with synthetic KiSS-1 peptide heightened adhesive properties of the cell and induced

changes in phosphorylated levels of P38, ERK and JNK. Addition of chemical inhibitors which block these different pathways resulted in loss of pathway activation and aberrant cellular activity and morphology, suggesting that each plays a major role in mediating the effects of KiSS-1.

During my Ph.D. research, I also determined two distinct and cell-type specific means whereby KiSS-1, like many metastasis suppressor genes, is lost during metastatic progression. Previous studies had indicated a correlation between reduced KiSS-1 expression and a loss of certain chromosomal regions. I identified that loss of two proteins, a transcription factor and a transcription co-factor, encoded by these regions caused the ensuing loss of KiSS-1 transcription in metastatic cells. Initially I identified two distinct AP-2α binding elements on the KiSS-1 promoter which mediated KiSS-1 transcriptional control and demonstrated that loss of AP-2α or targeted mutation of the two binding elements significantly reduced KiSS-1 levels in vitro. This was the first data to provide a mechanism for the loss of KiSS-1 during metastatic progression of breast cancer. Additionally, as KiSS-1 correlates directly to the degree of metastatic severity in human patients and its expression is dependent upon transactivation by AP-2α, this research also supported the use of AP-2α as a potential biomarker for identifying strong metastatic potential of certain breast cancers. A second, similar study looked at the effects of the transcriptional co-factor DRIP-130 on activation of the KiSS-1 promoter. A subunit of CRSP, DRIP-130 targets the common transcription factor, Sp1 to a promoter. My research indicated that activation of the KiSS-1 promoter, which contained multiple Sp1 sites, required DRIP-130 expression and that chromosomal loss of the region encoding DRIP-

130 resulted in abrogated transactivation of the promoter in highly metastatic melanoma. Using both EMSA and CHIP analysis as before in the AP-2α study, I was able to isolate the DRIP-130-modulated region of the KiSS-1 promoter and indicate a potential mechanism by which KiSS-1 is lost during progression of melanoma.

The last of my Ph.D. research focused on the spatial and temporal expression of the Rho family of GTPases in the mouse lens and their differential activation during lenticular differentiation. As the Rho family proteins are closely associated with cytoskeletal changes and cellular differentiation, I looked at possible roles of Rho regulation in normal and abnormal lens cell differentiation. Western analysis and immunohistochemistry suggested that activation of Rac1, a Rho protein associated with lamellapodia formation, correlated to periods of heightened lens differentiation. In order to examine the effects of Rac1 activation in vitro, I then developed a cell culture-based system in which I found that I could differentiate N/N1003A rabbit lens epithelial cells using a promiscuous guanine exchange protein which activated Rac1. In addition, I found that inhibition of Rac1 activation with a specific chemical inhibitor in both my cell culture system and lens explants resulted in loss of differentiation as gauged by decreased levels of β-crystallin and profilin, markers of lens differentiation. Immunofluorescence further revealed that activated Rac1 induced SRF nuclear localization, a transcription factor, which upregulates the expression of a β-crystallin promoter-driven luciferase construct and induces lens differentiation.

The results of this dissertation research have made significant strides in understanding the nature of the anti-metastatic effects registered by the novel KiSS-1 peptide and its cognate GPCR. Additionally, it has shed light on the Rho family regulation of lens epithelial cell differentiation, indicating the elaborate involvement of Rac1 in mediating lens fiber development. In all, this research has determined previously unknown roles of small molecule GTPases in both the progression of metastasis, as well as in normal and abnormal lens cell differentiation.

NOMENCLATURE

AAV	Adeno-Associated Virus
AP-2α	Activator Protein-2alpha
CDK	Cyclin Dependent Kinase
ChIP	Chromatin Immunoprecipitation
DRIP-130	Vitamin D Receptor Interacting Protein-130
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMSA	Electrophoretic Mobility Shift Assay
EtBr	Ethidium Bromide
FAK	Focal Adhesion Kinase
FGF	Fibroblast Growth Factor
GDP	Guanine Di-Phosphate
GEF	Guanine Nucleotide Exchange Factor
GPCR	G-Protein Coupled Receptor
GST	Glutathione S-Transferase
GTP	Guanine Tri-Phosphate
JNK	Jun N-Terminal Kinase
LPA	Lysophosphatidic Acid
MAPK	Mitogen Activated Protein Kinase
MLC	Myosin Light Chain
MMP	Matrix Metalloproteinase

NF-κB	Nuclear Factor-kappaB
PAK	P-21 Activated Kinase
PCO	Posterior Capsular Opacification
PDGF	Platelet Derived Growth Factor
PLC-β	Phospholipase C-β
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SP1	Specificity Protein 1
SRE	Serum Response Element
SRF	Serum Response Factor
TGF	Transforming Growth Factor
TGF-β	Transforming Growth Factor-β
VEGF	Vascular Endothelial Growth Factor

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